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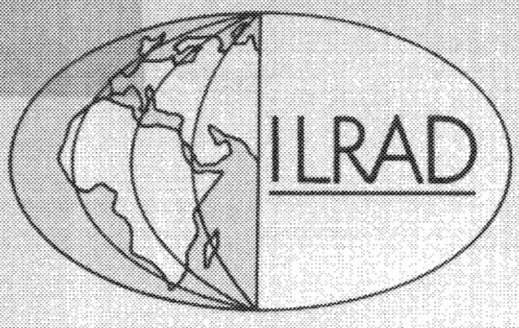

INTERNATIONAL LABORATORY FOR RESEARCH ON ANIMAL DISEASES

RESEARCH REPORT

1977

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INTERNATIONAL LABORATORY FOR RESEARCH ON ANIMAL DISEASES

RESEARCH REPORT 1977

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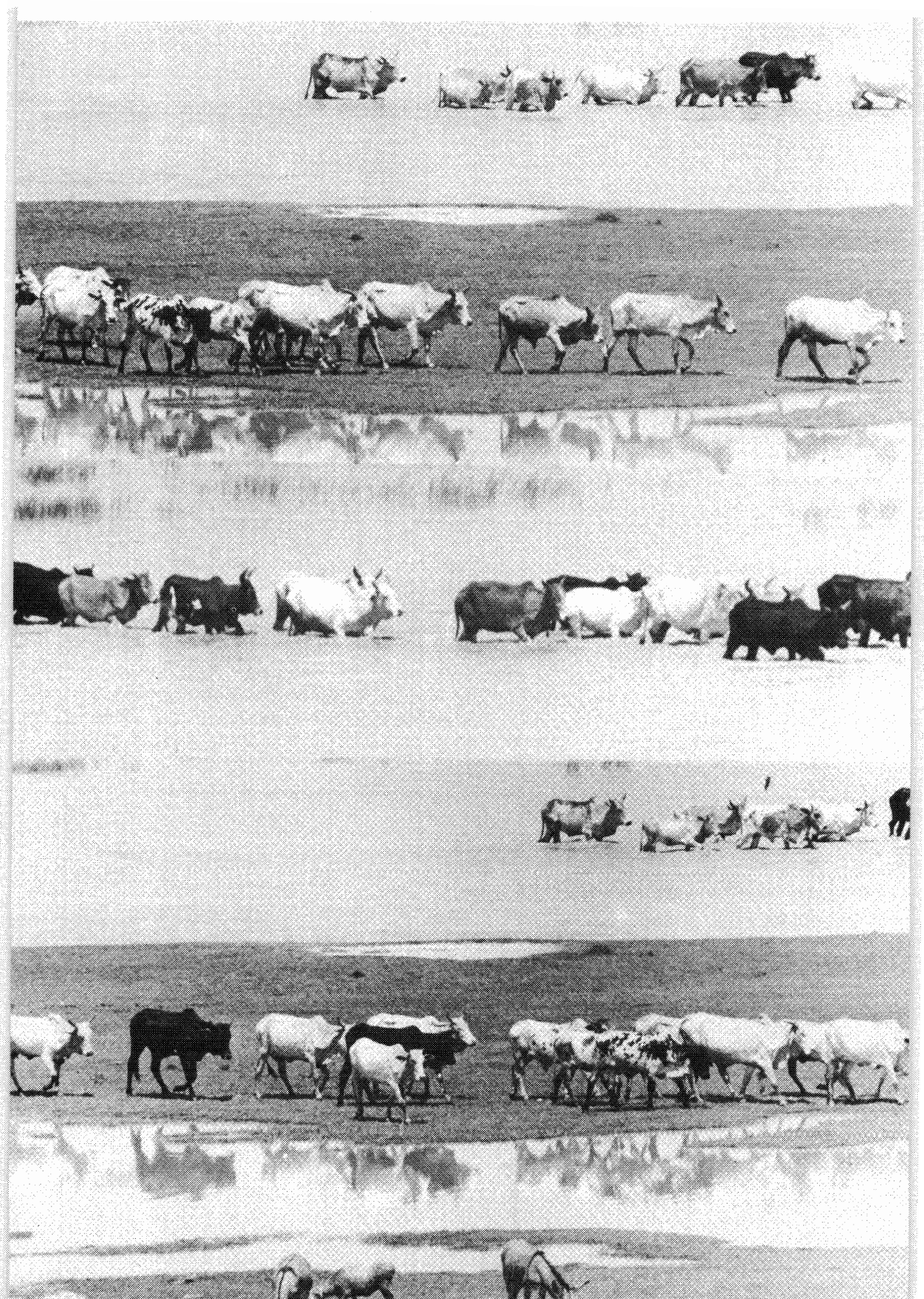

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Cover photograph: A Dinka cattle camp in the Southern Sudan. Duncan Willetts.

DIRECTOR'S REPORT

The mandate of ILRAD is to develop effective control procedures for trypanosomiasis and theileriosis, two diseases that constrain livestock production in a large number of countries in Africa, the Middle East, India and Central and South America. In addition to its focus on animal diseases, ILRAD is unique among the international agricultural research centres in also being potentially able to contribute to the control of important human diseases, such as trypanosomiasis, and thus the results accruing from the ILRAD programme will find application in both human and animal medicine. In addition, the disease mechanisms, the pathogenesis and potential control procedures are undoubtedly similar in many haemoprotozoal diseases of man and animals. As a result, ILRAD and its scientific staff are developing cooperative relationships with animal disease as well as human disease research institutions. This broadens the scope of expertise and potential cooperators that can address the problem.

The philosophy of ILRAD is to amalgamate basic and applied research to approach relevant questions leading to solutions to the two problems. The diseases are the guiding themes in all considerations. Due to the complexity of the maladies, it is essential that a multidisciplinary approach be utilized. A continuous feedback from the field must have an important role in guiding and appropriately orienting the laboratory activities to the 'real' problems in the field. ILRAD must also establish and maintain contact with national organizations in order that the results from the research programmes have the highest probability of valid field application. Inherent in this consideration is training of national staff with emphasis on the training of 'trainers'. Staff from national organizations through training, meetings, conferences, etc., should be brought together at ILRAD with ILRAD staff to forge lasting relationships. ILRAD is a unique institution that must be viewed and utilized as an international resource. Because of its location, make-up and mandate, ILRAD should serve as a focal point to bring together diverse activities relating to tropical diseases, training and field and laboratory research.

The overall establishment of ILRAD continued to progress in 1977. Additional scientific staff as well as support staff have been added. This has enabled an expansion in the scope of research and the refinement and augmentation of support activities. It is anticipated that the number of staff both in scientific and support categories will increase considerably in 1978, when the new facilities are occupied, to reach full staffing in late 1978 or early 1979.

The construction of the facilities has continued in 1977. Although the staff still occupy very cramped and widely scattered facilities, the research has continued to progress. At the time of writing, in early 1978, some of the scientists are already using the new laboratories with others soon to move. The inauguration and official opening of the institution by the Kenya Vice President and Minister for Home Affairs, the Hon. Daniel arap Moi, is scheduled for 5 April 1978. It is anticipated that at that time all of the facilities, except the dining centre and the hostel, will be finished and in use. Needless to say, the completion of the facilities, which will have occurred in slightly less than two years, is eagerly anticipated by ILRAD staff.

The research activities made very notable advances in 1977 as evidenced by the short descriptions of the research which follow. In addition, the reader is referred to the list of scientific papers submitted for publication. In order for the results to be of maximum benefit to others interested and involved in trypanosomiasis and theileriosis, it is imperative that the results be published quickly and be as accessible as possible to scientists throughout the world. ILRAD emphasizes the rapid publication of research

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results in scientific journals, with only a brief synopsis of the activities described in this publication.

Continued expansion of the trypanosomiasis programme has occurred in 1977. The *in vitro* propagation of the bloodstream forms of trypanosomes, which were described in 1976, has been expanded to include the insect forms as well as the ability to clone individual trypanosomes. The former will in the future make it possible to grow metacyclic trypanosomes in culture for use in a number of important research approaches directed to elucidation of metacyclic antigens and their potential use in immunization. The latter offers the opportunity to carry out investigations on genetically homogenous material and opens up avenues for studying a number of aspects of antigenic variation and other important considerations in the disease. Research on the antigens and membrane components of trypanosomes has likewise been continued with the description for the first time of a common determinant in or on the variant specific surface antigen. This research will make it necessary to re-examine the question of the make-up of the variant antigen and suggests that there may be common and variable determinants similar to those observed in the immunoglobulin system. This requires further elucidation, however. The role that the common determinant(s) play in the pathogenesis and eventual control of the disease remains to be proved. Research on the pathogenesis of the disease has progressed, utilizing materials from animals that have been experimentally infected in Nairobi, as well as from field experiments carried out in West Africa.

The immunology of African trypanosomiasis is relatively unknown and appears to be complex. There is a hyperresponse directed against some trypanosome antigens, yet the usual host defence mechanisms are incapable of ridding the animal of the organism. Further work, described later in this document, indicates research activities being carried forward which are of fundamental importance to an understanding of the immunology of African trypanosomiasis. Inherent in the study is an understanding of the immunology of the bovine immune system. Knowledge of the bovine immune system is incomplete and is required to be able to understand the function and functional deficiencies of the system in trypanosomiasis and other diseases.

Field work in the Gambia has demonstrated that Ndama cattle are more resistant than Zebu cattle to needle challenge with bloodstream forms of trypanosomes and to tsetse fly challenge. The mechanisms involved are of fundamental importance in efforts to immunize against African trypanosomiasis and are being pursued.

The theileriosis research has also gained impetus during 1977. This work is continuing at ILRAD in cooperation with other institutions in East Africa and elsewhere. Activities have included the planning, initiation of construction and at the time of this writing almost the completion of the tick and stabilate facility which should provide needed space for work on the tick vector and causative agent of East Coast fever.

A great deal of work has been carried out on the *in vitro* propagation of theileria. Included is the use of bovine lymphoid cells as well as efforts to grow preschizont stages of the parasite in tick and mammalian cell cultures. These efforts provide material for other research and attempt to attenuate, by cell culture means, theileria for potential immunization. Initial stabilate material for this and other work has been kindly supplied by our colleagues in the Kenya Veterinary Research Department (formerly EAVRO).

Research has also been carried out to demonstrate theileria antigens specific for given isolates and for use as potential immunogens. This work is continuing to examine the surface of infected lymphocytes as well as look at the antigens in macroschizonts and piroplasms. Other investigations have suggested that the cells supporting theileria replication are T lymphocytes.

Several stabilates have been prepared and titrated in cattle. Ticks are also being propagated for the work. In addition to the tick work at ILRAD, cooperative research has been under way in conjunction with the Department of Parasitology and Tropical Veterinary Medicine at the Frei University of Berlin on the selection of hypersusceptible *Rhipicephalus appendiculatus* ticks, the vector of East Coast fever. Cooperative activities with the theileriosis group at the Kenya Veterinary Research Department at Muguga have also expanded.

In 1977 ILRAD, in cooperation with the OAU, WHO and FAO, hosted a Leadership Training Course in Trypanosomiasis with notable success. Representatives from over 30 African countries were brought together in Nairobi to learn about recent advances in human and animal trypanosomiasis and about tsetse-related activities, to undergo field and laboratory training, to plan trypanosomiasis control programmes and to meet colleagues from surrounding countries. The course was conducted by visiting instructors as well as ILRAD and other East African institution staffs. Additional training activities included individual and small group training, graduate student, post-doctorals, seminars and courses.

In order for ILRAD to most effectively utilize its resources, the institution must have a method of continual assessment of research priorities and progress, by both internal as well as external review. The ILRAD staff and the Programme Committee of the Board provide the internal evaluation. A Scientific Advisory Committee composed of eminent, internationally recognized scientists who do not have a direct relationship with ILRAD was convened to examine independently the institution, its programmes, staff, facilities and research progress. This group, as well as the internal evaluations, has indicated

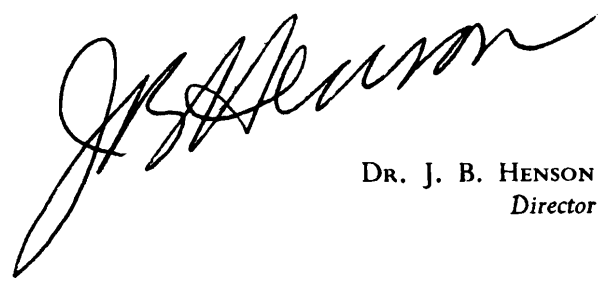
that ILRAD had made significant progress and was 'on target'. This review process, which will continue, enables ILRAD and its staff to gain from the view, insight and experience of scientists from outside the institute. External and internal review assist in our establishing priorities and assessing progress.

A need for a better source of appropriate quality cattle, which is one of the cornerstones of the research activities, was noted by both the external and internal reviews. Approaches to solving this problem have been included in the revised Programme and Budget document for 1978 and 1979. This is an extremely important consideration for the institute and must receive a high priority.

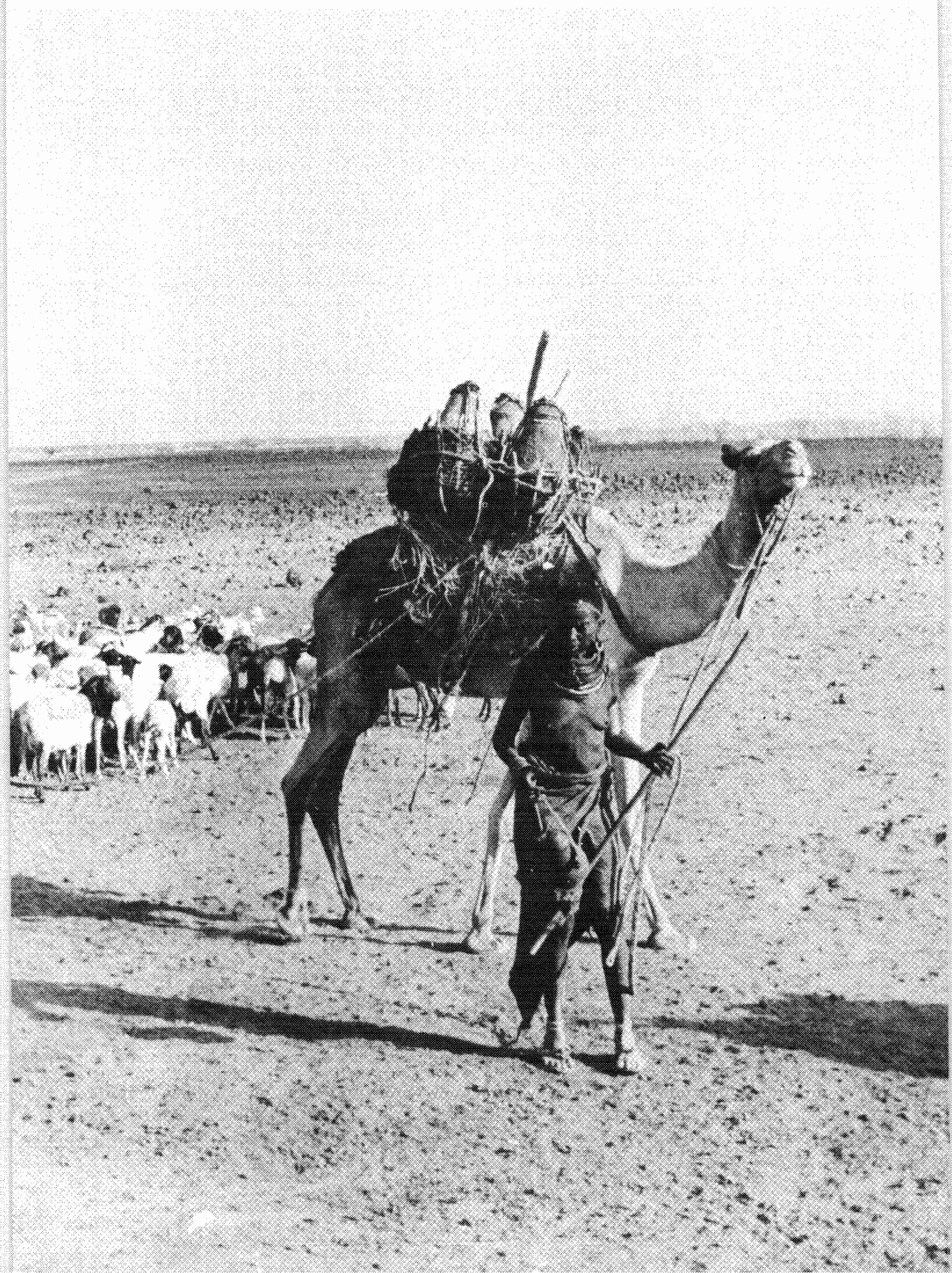
This, the second ILRAD Research Report, is the last one to be prepared by the present Director. I have resigned effective 1 July 1978, with considerable regrets. This document indicates the continued growth and development of ILRAD. From my biased vantage point, I feel that excellent progress has been made and that the potential contribution by ILRAD in a number of activities is great. Likewise, I feel that the whole Consultative Group for International Agricultural Research (CGIAR) system has great prospects for making meaningful contributions to food production in the developing countries. Successes have already occurred about which we are all familiar. The *modus operandi* of the International Agricultural Research Centres and the CGIAR offer clear-cut methods and approaches to problems minimally encumbered by bureaucracy and red tape. Let us hope such continues to be the case.

ILRAD, as one of the International Centres, is different from the others in the scope of its mandate and the type of research required. One must always keep in mind the foundation of information upon which all the International Centres' activities are based. Because ILRAD conducts activities on animal disease, while most of the other centres are primarily plant oriented, evaluation of the success or lack of success of ILRAD must be viewed and evaluated from a slightly different perspective. As an example, *in vitro* propagation of trypanosomes does not have direct field application but opens many doors leading to other necessary research, possible application to drug development, etc. This is a success. Other examples could be cited as well. It is important that these and other relevant considerations be kept in mind when ILRAD is viewed by the Technical Advisory Committee (TAC) and the donors.

I would like to take this opportunity to thank TAC, the CG Secretariat, and donors for their support in these early developmental stages of ILRAD. This confidence and support have enabled the institution to move quickly and become productive within a relatively short time. The ILRAD staff likewise have my thanks for their efforts over the last two years. I am sure that their work and dedication to ILRAD, its principles and philosophy, during these formative years will chart a course destined for success. ILRAD is unique; there will probably never be another institution like it. It will succeed.



DR. J. B. HENSON
Director



TRYPANOSOMIASIS

Trypanosomiasis occurs in a number of domestic animal species including cattle, swine, sheep, goats, horses and camels—and in man. It is caused by several species of blood parasites of the genus *Trypanosoma*. Included are *T. congolense* and *T. vivax*—the most important trypanosomes causing disease in cattle. Trypanosomiasis is transmitted by the tsetse fly and has wildlife reservoirs. The disease does occur, however, in areas where the tsetse fly is not present and in these cases is transmitted by biting flies. Trypanosomiasis is observed in many countries in Africa and in the northern areas of South America. In Africa, over most of the large area lying approximately between latitudes 16°N and 10°S, cattle raising is either precluded or is greatly reduced due to the disease. Paradoxically, this area experiences some of the highest rainfall on the African continent and has some of the greatest potential for forage production.

Trypanosomiasis adversely influences animal production in several ways. Some animals die fairly quickly after contracting the disease, while others may develop chronic infections which may last for months or years, with infected animals in poor body condition, giving poor reproduction and inefficient utilization of nutrients.

ILRAD research on trypanosomiasis is directed to studies of the parasite, the host and the host-parasite interactions including immunology and pathogenesis of the disease. Much is unknown and much information is required concerning these general areas. Field work, primarily in West Africa, is also being carried out and is providing much needed data on the field occurrence of the disease and on trypanotolerance. Consideration is being given to the possible prevention of infection and to enabling animals, once infected, to be tolerant. More details will be given in the following synopses and in the research papers given in the section on publications.

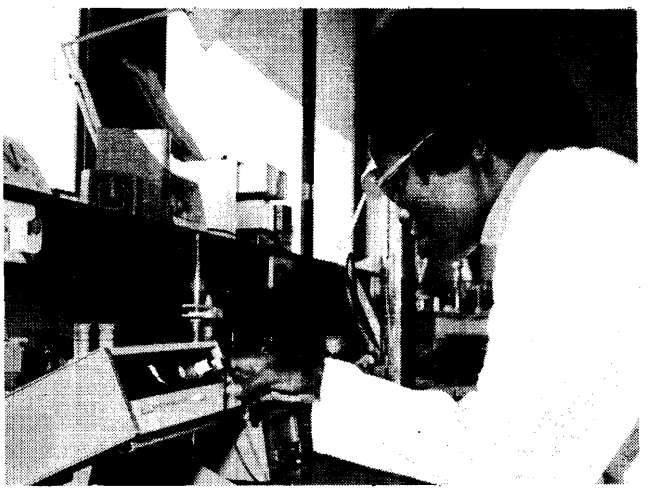
PATHOGENESIS OF AFRICAN TRYPANOSOMIASIS

Anaemia

A haemolytic factor has been identified in association with *T. congolense*, *T. vivax*, *T. brucei*, *T. gambiense* and *T. rhodesiense*. This factor would appear to be a heat-stable protein with a molecular weight of around 10,000 daltons. This activity has been found in trypanosome incubates, fresh freezed-thawed intact trypanosomes and purified variant surface antigen. Haemolytic activity has been demonstrated in the plasma of infected mice, rats and cattle, and this reactivity has been fractionated from the infected plasma. Further studies are under way to investigate the nature of this factor and its mode of action and to evaluate its possible role *in vivo*.

In collaboration with Glasgow University and Dr. Jim Dargie, pathophysiological studies on the erythropoietic response were carried out in *T. congolense*– and *T. brucei* – infected Zebu and Ndama cattle using ^{51}Cr-labelled blood cells and ^{59}Fe-labelled transferrin. No major difference in erythropoietic responsiveness was found between breeds, the only differences being the result of variation in the degree of anaemia between the breeds. This in turn was directly related to the level of parasitaemia. It was found that there was an increase in red blood cell breakdown (table 1) which was mainly the result of erythrophagocytosis reflecting a haemolytic component of the anaemia. In addition, it was considered that the haemopoietic response was not what might have been expected for the level of anaemia present and there was some indication of impairment of red cell production. Furthermore, there was evidence that iron trapping was occurring in the mononuclear phagocytic system and there was an apparent inability to reutilize much of the constituent iron for further haemoglobin synthesis. The results obtained for *T. congolense* and *T. brucei* were similar. We feel that the mechanisms responsible for the anaemia and the kinetics of the anaemia must vary during the course of the disease. The present study was carried out from 8 to 16 weeks postinfection.

These findings confirm pathologic observations made in acute and chronic cases of trypanosomiasis,



Dr. Rachel Masake measuring the pH of dilution medium used in suspending blood for the capillary concentration method used in the detection of all species of trypanosomes.

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TABLE 1. ^{51}Cr red cell survival and plasma iron kinetics (mean ±SE)

	^{51}Cr RBC $t_{\frac{1}{2}}$ (days)	Plasma iron pool (mg/kg)	Plasma ^{59}Fe $t_{\frac{1}{2}}$ (mins)	PITR (mg/kg/day)
Ndama:				
Infected	9.3±0.7	0.562±0.035	79± 5.9	0.75±0.09
Control	14.6±0.5	0.474±0.035	129±20.6	0.36±0.06
P	<0.01	not significant	<0.02	<0.05
Zebu:				
Infected	8.3±0.6	0.728±0.072	73±5.6	1.00±0.09
Control	14.0±0.4	0.475±0.039	133±7.5	0.36±0.05
P	<0.001	<0.05	<0.001	<0.002

PITR=plasma iron turnover rate

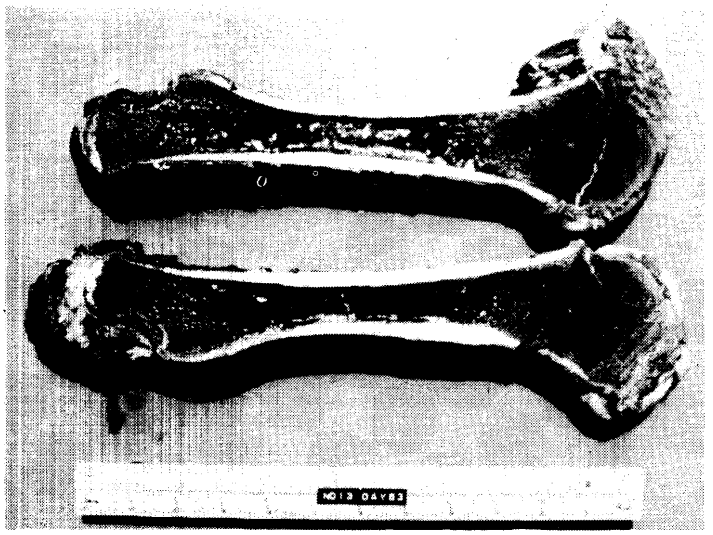

Figure 1. Longitudinal section of femur of Zebu infected with *T. brucei* for 53 days. There is a significant haemopoietic response.

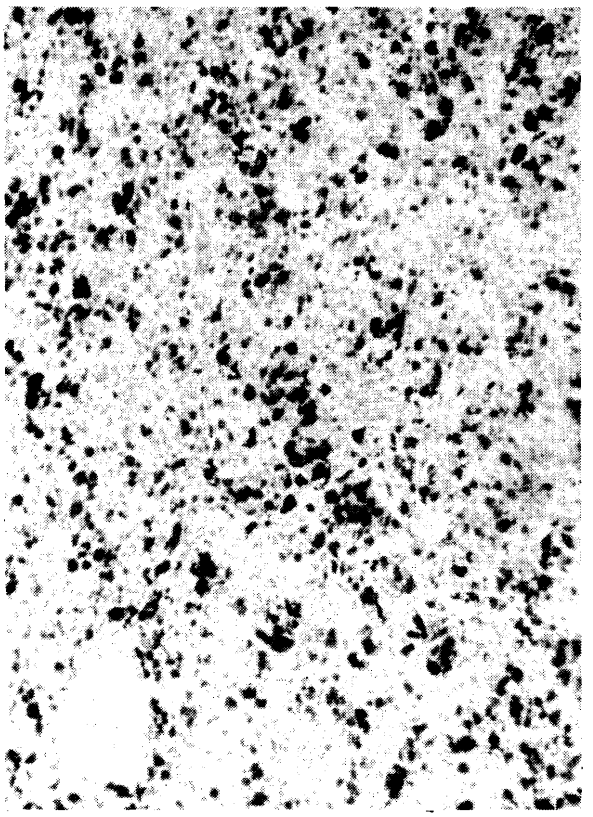

Figure 2. External haemosiderin deposits in spleen of Zebu chronically infected with trypanosomiasis.

Figure 3. Longitudinal section of femur of Zebu chronically infected with trypanosomiasis. The marrow is yellow and unreactive.

namely that in the acute phase of the disease the anaemia has an important haemolytic component with erythrophagocytosis, splenomegaly and marrow erythropoietic response (fig. 1). In chronic cases while erythrophagocytosis is still evident, splenomegaly is not; haemosiderosis is marked, confirming Fe trapping (fig. 2), while the bone marrow is yellow and aplastic (fig. 3).

Studies using ^{121}I-albumin and ^{51}Cr red blood cells demonstrated a significant increase in plasma volume in trypanosome-infected Zebu and Ndama, while circulating red cell volumes were significantly reduced. In all cases the total blood volume was unaltered by infection. Thus estimation of anaemia by packed cell volume gives an accurate estimation of the circulating red cell volume.

Pathology

In-depth histopathological, histochemical and immunopathological studies are ongoing in cattle, dogs and mice experimentally infected with *T. congolense*, *T. brucei* and also in mice infected with *T. vivax*. The material collected was from terminal

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Figure 4. Reactive lymph node of Ndama infected with *T. congolense* for four weeks.

Figure 5. Depleted lymph node of Ndama with long-standing infection of trypanosomiasis.

cases and at various intervals after infection, and also from animals slaughtered sequentially in order that the course of pathological changes could be followed. Special emphasis is being placed on lymph nodes (figs. 4 and 5), spleen, thymus, bone marrow and blood. Work in the dog is in collaboration with the Faculty of Veterinary Science, Nairobi, and the Kenya Veterinary Laboratories.

Diagnosis of African Trypanosomiasis

Studies have been continued on improvement of parasitological diagnostic techniques and the factors affecting blood sampling for anaemia and parasitaemia in bovine trypanosomiasis. These studies were carried out in collaboration with Glasgow University Veterinary School. It was found that the buffy coat–darkground phase technique detected the trypanosomes to a level of 10^2 trypanosomes/ml; in our hands this was 1 to 2 $\log_{10}$/ml superior to other parasitological microscopical techniques. In addition, it allowed species identification of trypanosomes on the basis of size and behavioural pattern.

It was found that blood samples from ear veins gave significantly higher packed red cell volume (PCV) values than did jugular samples; differences were greatest in the early morning, at which time ear vein samples showed greater variation. Both ear vein and jugular samples registered a small peak in PCV values around mid-day, but otherwise jugular samples showed no significant diurnal variation. There was no clear diurnal parasitaemic pattern, but there was some evidence to suggest that samples taken around mid-day are more likely to reveal the existence of infection. Ear blood was more sensitive than jugular blood in the detection of parasitaemia.

MAX MURRAY
W. I. MORRISON
L. ROVIS

TRYPANO-TOLERANCE

The exploitation of trypano-resistant or trypano-tolerant livestock in areas of Africa dominated by the tsetse fly is regarded as offering a potential solution to the problem of trypanosomiasis. However, to date little is known about the extent or basis of trypano-tolerance. The present series of ongoing experiments in cattle and in mice represent part of a programme undertaken to unravel the secrets of this phenomenon.

Studies in Cattle

In collaboration with Glasgow University Veterinary School, the Gambian Government and the Rockefeller

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Foundation, we examined the comparative susceptibility of Ndama cattle (trypano-tolerant) and Zebu cattle (trypano-susceptible) to needle challenge with *T. congolense* and/or *T. brucei* and to challenge with the tsetse fly *Glossina palpalis gambiense*. In addition, the effect of weight of challenge on the outcome of the disease process was considered.

The study, which lasted for 411 days, showed a dramatic difference in susceptibility between the two breeds following both needle and fly challenge. Of the 40 Zebu left to die, 30 died of trypanosomiasis, whereas none of the 37 Ndama left to die died of trypanosomiasis. Eight Zebu died following needle challenge, while the remainder died during the period of tsetse fly challenge.

The cardinal clinical sign and major disease-promoting factor of African trypanosomiasis in cattle is anaemia. In this study, while both breeds became anaemic and ill, the degree of anaemia that developed in the Zebu was much greater than that in the Ndama. During the period of tsetse fly challenge, the Zebu remained anaemic while most of the Ndama were apparently unaffected. This was due to the fact that only a few of the Ndama became reinfected at this time while all of the Zebu did. On the other hand, both groups were susceptible to reinfection by needle challenge. By the end of the experiment, two-thirds of the Ndama had recovered as had three of the ten Zebu survivors, as judged by PCV and clinical condition. The remaining Ndama and Zebu had PCV values of 27% or less and were considered as still being anaemic.

These changes and differences in anaemia were apparently directly related to the different ability of the two breeds to limit the levels of parasitaemia. While following needle challenge, little difference emerged between prepatent period and time to first peak of parasitaemia, major differences were found between the level and duration of parasitaemia (tables 2 and 3). In all cases the Ndama showed superior ability to limit the level of the first and subsequent peaks of parasitaemia and also the duration of parasitaemia. All Ndama and ten Zebu survivors were able to eliminate the trypanosome, thus affecting self-cure, in most cases several months before termination of the experiment. This was assessed by intensive parasitological examination of blood and tissues. It is important to emphasize that the parasitaemic profile in a trypanosome-infected bovine is quite different from that in a trypanosome-infected

TABLE 2. Parasitaemia profile in Zebu and Ndama cattle challenged with 2.6×10^8 *T. brucei*. Tsetse fly challenge on day 155

	Zebu	Ndama
Prepatent period in days	2	2
Time to first peak of parasitaemia in days	4	4
First peak of parasitaemia estimated as plus score	5.00	3.78
Average duration of parasitaemia in days	218	105
Range of duration of parasitaemia in days	68–289	28–270
Duration of study in days	411	411

Source: C. M. Murray et al. 1977.

TABLE 3. Parasitaemia profile in Zebu and Ndama cattle challenged with 3.5×10^7 *T. congolense*. Tsetse fly challenge on day 59

	Zebu	Ndama
Prepatent period in days	4	4
Time to first peak of parasitaemia in days	10	13
First peak of parasitaemia estimated as plus score	4.81	4.00
Average duration of parasitaemia in days	193	111
Range of duration of parasitaemia in days	193	34–206
Duration of study in days	315	315

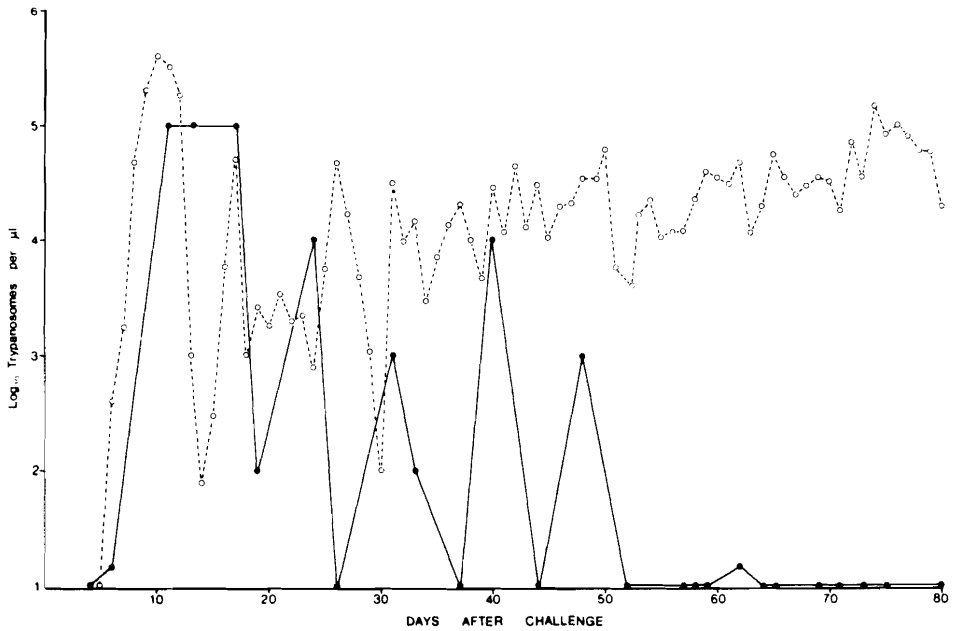

Figure 6. The levels of parasitaemia recorded in a resistant C57Bl/6J female mouse (- - O - -) and a trypano-tolerant Ndama female bovine (—●—), both inoculated with *T. congolense*. The C57Bl/6J mouse died on day 123 postinoculation whereas the Ndama had self-cured by day 65 and remained negative until the experiment was terminated on day 315.

Figure 7. Ndama with sleeping sickness.

mouse, even a mouse of a resistant strain. Once a mouse is infected, parasitaemia is ongoing until death, whereas cattle, especially Ndama, have the ability to limit the parasitaemic levels and eventually eliminate the parasite (fig. 6).

When death occurred in the Zebu cattle it was due to acute congestive heart failure which was the result of anaemia and associated myocardial damage. While *T. congolense* was more pathogenic than *T. brucei*, in the present study both Zebu and Ndama infected with *T. brucei* became ill and anaemic and two Zebu died.

While clear differences emerged between Zebu and Ndama following both needle and tsetse fly challenge, it should be emphasized that the Ndama also became anaemic. Several aborted during the early stages of infection. As part of this study, the effect of dose on responsiveness was examined. It was found that animals receiving the highest challenge doses became most anaemic, the level of parasitaemia was higher, and the duration of parasitaemia, at least in Ndama, was longer. Only the first part of the experiment could be analysed in this way because all animals came under tsetse fly challenge during the latter part of the study. As well as demonstrating a dose response effect, these studies showed that trypano-tolerance was not absolute but was only relative and depended on weight of challenge. Thus it was found that the Ndama receiving the highest challenge dose were as anaemic as Zebu receiving the lowest challenge. This was confirmed in a small study in which five yearling Ndama were inoculated with 1×10^9 *T. congolense;* all cattle became anaemic (fig. $\frac{1}{2} \times 10^9$), showing a marked reduction in condition and weight. Four of the five Ndama died.

The basis of trypano-tolerance has yet to be understood. The present findings indicate that it is related to the ability to control the level and duration of parasitaemia, which in turn influence the severity of the anaemia. It is our hypothesis that trypano-tolerance is related to the capacity to mount an effective immune response. Present investigations involve a study of the histopathology of trypanosomiasis in both Ndama and Zebu, with special reference to the lymphoid system and also a comparison of immune responsiveness to the trypanosome per se.

Studies in Mice

In parallel with studies in cattle, we have examined the susceptibility of different inbred strains of mice to *T. congolense* infection. Having established that differences in susceptibility occur between strains of mice, we have made an attempt to determine the underlying mechanism and to characterize its genetic basis.

T. congolense infection was compared in eight inbred strains of mice. Marked differences in survival were found between the eight strains, ranging from the highly susceptible A/J, all of which had died by day 30 after infection, to the much less susceptible C57Bl/6, most of which survived beyond day 80 (table 4). There was not a clear-cut differentiation into strains of high and low susceptibility but rather a gradation between the A/J and C57Bl/6 strains. A similar pattern of relative susceptibility has been found

TABLE 4. Duration of survival in inbred strains of mice infected with *T. congolense*

Mouse strain	Percentage deaths (days after infection)					Mean survival time (days)
	20	40	80	120	160	
A/J	85.7	100.0				15.8
SWR/J	88.0	100.0				16.9
129/J	58.5	90.2	100.0			22.6
BALB/c/A	20.0	28.8	100.0			49.5
DBA/1J	0.0	62.2	100.0			36.3
C3HeJ	0.0	12.5	100.0			59.0
AKR/A	0.0	7.9	45.4	97.7	100	81.7
C57Bl/6	0.0	2.8	13.8	61.1	100	110.2

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TABLE 5. Suppression of response to mitogens and allogeneic cells (MLC) and suppressor activity (*) on normal MLC of spleen cells from four strains of mice infected with *T. congolense*

Mouse strain	PERCENTAGE SUPPRESSION							
	Day 9		Day 17		Day 24			
	Con A	LPS	Con A	LPS	Con A	LPS	MLC	Suppressor*
A/J	95	90	100	100	100	95	100	100
C3H/Tif	98	88	99	100	96	100	91	98
AKR	87	86	81	71	82	100	69	56
C57Bl/10	13	4	87	80	91	82	52	38

using 5 other isolates of *T. congolense*. However, the differences in susceptibility were very much dependent on the virulence of the trypanosomes. Thus, use of a relatively virulent trypanosome caused much smaller differences in survival between strains, while there were also *T. congolense* isolates of low virulence that produced infections in excess of 30 days in duration in A/J mice. Susceptibility was closely related to the levels of parasitaemia, at least during the early stages of infection. Thus, in A/J mice consistently high levels of parasitaemia in excess of 10^5 organisms per μl were found from day 9 onwards. By comparison C57Bl/6 mice showed a characteristic series of parasitaemic peaks between which the level of parasitaemia was extremely low or undetectable. C3H/He mice, which were of intermediate susceptibility, initially showed a pattern of parasitaemia similar to C57Bl/6 but later showed sustained high levels of parasitaemia.

Infectivity titrations carried out in A/J and C57Bl/6 mice have shown similar ID_{50} values for the strains. Thus there does not appear to be a difference in susceptibility to infection per se but rather a difference in the ability of the different strains of mice to limit the numbers of parasites in the circulation.

To test the effects of infection on the immune system of four of the mouse strains (A/J, C3H/Tif, AKR and C57Bl/10) spleen cell suspensions, prepared from infected and control mice, were tested for response to the mitogens lipopolysaccharide (LPS) and Concanavalin A (Con A) and in mixed lymphocyte reaction (MLR) with allogeneic spleen cells. In addition, the suppressor activity of infected spleen cells on an ongoing MLR of syngeneic control responder cells with allogeneic targets was tested. The strain of *T. congolense* used was found to be slightly more virulent than that used in the initial studies, so that the C3H/Tif mice showed higher levels of parasitaemia and died at an earlier stage of infection than the C3H/He mice in previous experiments. However both AKR and C57Bl/10 mice showed long-standing infections. In general the response to Con A and LPS was more depressed in the strains of highest susceptibility. With the exception of C57Bl/10 mice at day 9, depression was always greater than 70%. At day 24 of infection, both the depression of response of infected spleen cells in MLR and their suppressor activity on normal MLR appeared to be related to mouse strain susceptibility. That is, spleen cells from the more susceptible strains showed greater depression and suppressor activity (table 5).

Among the eight strains of mice examined, no obvious association between H-2 haplotype and susceptibility was apparent. However, to examine this critically a comparison of susceptibility was made between C57Bl/10 mice and the congenic resistant strains B10A, B10.BR and B10.D2/new. No significant differences in levels of parasitaemia or in survival were found. Thus it would appear that the H-2 haplotype does not obviously influence susceptibility to *T. congolense* infection.

A comparison of susceptibility was also carried out in A/J and C57Bl/6 mice, A/J × C57Bl/6, F1 hybrids and the corresponding backcrosses. This experiment is still in progress and is now at day 60 after infection. All of the A/J and 20% of the (F1 × A/J) mice have died, while no deaths have occurred in the other groups. All of the F1 hybrids and the F1 × C57Bl/6 backcross mice showed a cyclical pattern of parasitaemia similar to that of the C57Bl/6. However the

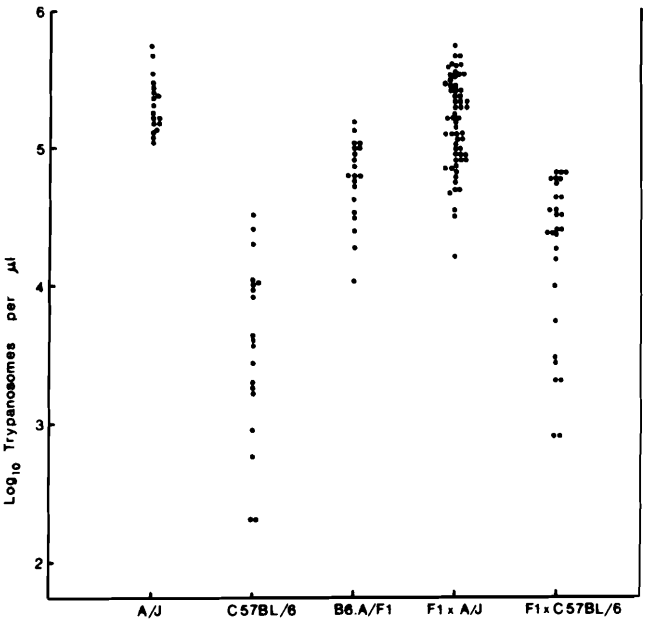

Figure 8. First peak of parasitaemia in individual A/J, C57Bl/6 F1 hybrid and backcross mice infected with *T. congolense*.

peak levels of parasitaemia in the F1 hybrids were significantly higher than in the C57Bl/6. The F1 × C57Bl/6 backcross mice generally showed a level of parasitaemia intermediate between the parental strains (fig. 8). The levels of the initial peak of parasitaemia in F1 × A/J backcross mice were found to overlap those found in the A/J and F1 hybrid mice. As in the A/J parents, in some of the backcross mice this high level of parasitaemia was maintained. However, in approximately 70% of the animals the level of parasitaemia dropped dramatically by at least 2 $^{\log}10$ units before rising to a second peak. Thus it would appear that the relative resistance exhibited by the C57Bl/6 mice is inherited as a partially dominant trait, under complex genetic control involving at least two genes.

Preliminary studies indicate that there are marked differences in total immunoglobulin subclass responses and in mononuclear phagocytic function between A/J and C57Bl/6 mice infected with *T. congolense*. It is therefore proposed to extend these studies and to examine in detail the subclass specific anti-trypanosome responses in the different strains.

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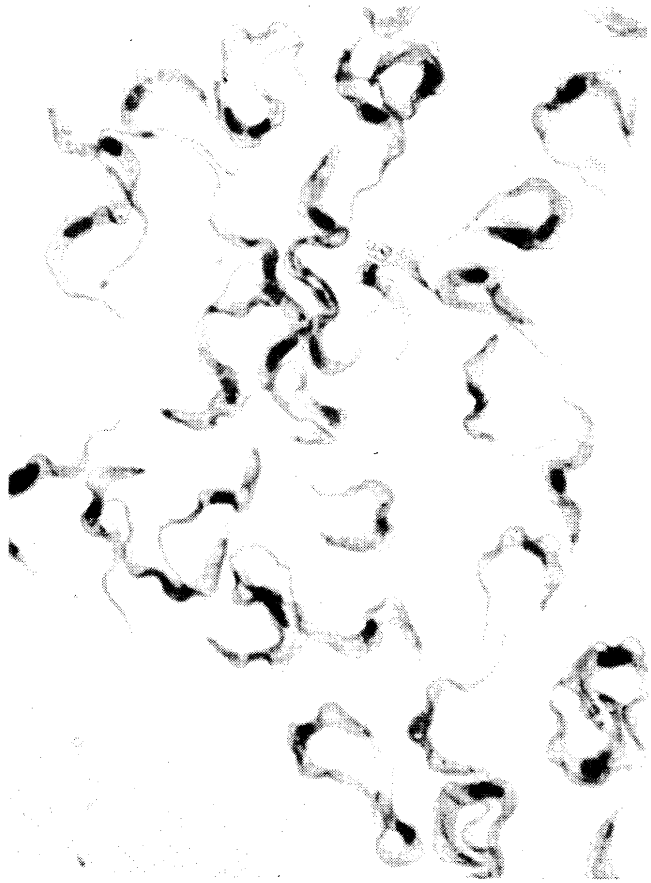

Cultured trypanosomes—bloodstream form.

IN VITRO PROPAGATION OF TRYPANOSOMES

During the past 70 years, attempts to cultivate animal-infective forms of salivarian trypanosomes for any significant period of time have met great difficulties. This inability to grow the animal-infective forms in the laboratory has hindered trypanosomiasis research significantly.

During the last two years our major emphasis, therefore, has been placed on the establishment and standardization of *in vitro* systems that support (1) the long-term propagation of animal-infective forms, (2) cloning of the cultured bloodstream forms and (3) the reproduction of the complete life cycle.

Long-Term Cultivation

A total of 96 different culture conditions for *T. brucei* (Strain 427) were tested. The best growth rate was obtained with HEPES buffered RPMI 1640 medium containing 20% heat-inactivated foetal bovine serum at pH 7.2, osmolarity 290 mosm, at 37°C.

The organisms grown in this system were morphologically identical to the long-slender bloodstream forms grown *in vitro*, retained infectivity for mammalian hosts (cattle and mice) and displayed variable antigen on their surface. The organisms maintained *in vitro* have these characteristics for over 20 months.

Extensive investigations of their growth dynamics *in vitro* were made, and the culture techniques have been standardized. Bloodstream forms can now be easily monitored *in vitro* for prolonged periods of

Mrs. Hirumi subculturing trypanosomes as part of the cell culture programme.

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time. Recently, long-term cultures of a different strian, *T. brucei* (Strain 227), have also been made.

In vitro Cloning

Laboratory techniques of cloning of the cultured bloodstream forms *in vitro* have been developed. A total of 18 clones (three different antigenic types) were successfully established.

An antigenic type, tentatively designated as type 221, seemed to be stable *in vitro* for over 10 months. The stability of the other two types, C52 and 227, is presently under examination.

The growth rate of 221 was significantly higher than that of C52. In cultured populations that were experimentally mixed with a single 221 and 10^5 C52 trypanosomes, 221 trypanosomes became predominant (over 99.99%) by day 10. Further results on antigenic variation of cultured bloodstream forms will be discussed elsewhere in this report.

Life Cycle in Culture

The life cycle of *T. brucei* has been completely reproduced *in vitro*. Cultured bloodstream forms transformed to insect midgut forms when they were transferred from 37°C to 25°C and became non-infective for mice by day 12–14. On day 25 and onwards, epimastigotes and metacyclic forms were seen in the cultures. The populations containing the metacyclic forms were infective for mice. The organisms in such populations rapidly retransformed to the bloodstream forms within 8 days after they were again transferred to 37°C.

A second life cycle was also achieved by repeating the same procedures. The bloodstream forms that underwent the complete life cycle *in vitro* demonstrated their original antigenic type 221.

Biochemical studies of specific surface antigens of cultured trypanosomes are presently under way with the collaboration of L. Rovis.

Future Plans

1) Attempts will be made to establish long-term cultures of newly isolated strains of *T. brucei* as well as of *T. congolense* and *T. vivax*.
2) Further studies on the mechanism of antigenic variation and biochemistry and immunology of the antigens will be made with collaboration of biochemists and immunologists.
3) Extensive studies on the nutrient requirement (amino acid analyses and carbohydrate metabolism) of the trypanosomes in culture will be carried out, aiming for the development of host cell-free systems, serum-free media and large-scale culture systems.
4) Attempts to attenuate the bloodstream forms by the prolonged cultivation and/or by chemical treatments (ethidium bromide, etc.) *in vitro* will be made. If the attenuation is achieved, their protective antigenicity in host animals will be investigated.

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MORPHOLOGICAL AND PHYSIOLOGICAL STUDIES ON *TRYPANOSOMA (NANNOMONAS) CONGOLENSE*

The pleomorphism of bloodstream trypanosomes of the *T. brucei* species, with its related physiological changes, is a well-recognized phenomenon. It results from physiological/biochemical changes within the trypanosomes during the course of each parasitaemic wave, which both limit the parasite's multiplication within the mammalian host and ensure its successful adaptation to the conditions present in the tsetse fly.

While pleomorphism in *T. congolense* has been recognized for some considerable time, this observation initially resulted in the classification of two separate species, *T. congolense* and *T. dimorphon*, on the basis of parasite length. Subsequently these were reclassified as one species, *T. congolense*, but the species was considered to comprise strains that were typically short (*congolense*), intermediate, and long (*dimorphon*) types. Such investigations had in general been carried out using parasites derived from animals in various parts of Africa.

In our studies pleomorphism of bloodstream *T. congolense* was observed during the course of a parasitaemic wave in the mouse using cloned and uncloned derivatives of four recent isolates of East African origin. It was found that at any point of parasitaemia there were several morphological types of the parasite, ranging from short to long forms. In the rising phase of parasitaemia, however, the appearance of the parasites was monomorphic and the short forms predominated. At peak parasitaemia the parasites were highly pleomorphic (fig. 9) with significant proportions of short and intermediate forms, although the long forms predominated. These different morphological types of the parasite also differed in their ability to infect a new mammalian host (fig. 10 and table 6).

Pleomorphism was observed both in normal and in lethally irradiated (900 R) mice, even when the infection was initiated using a single organism. Such pleomorphism may result from physiological differences between the different forms of this parasite in a manner similar to the morphological/physiological relationships already described in *T. brucei brucei*.

Further investigations were then carried out to determine standard conditions under which tsetse flies, *Glossina morsitans*, could be infected with this parasite. High infection rates were consistently achieved when teneral flies, maintained under defined

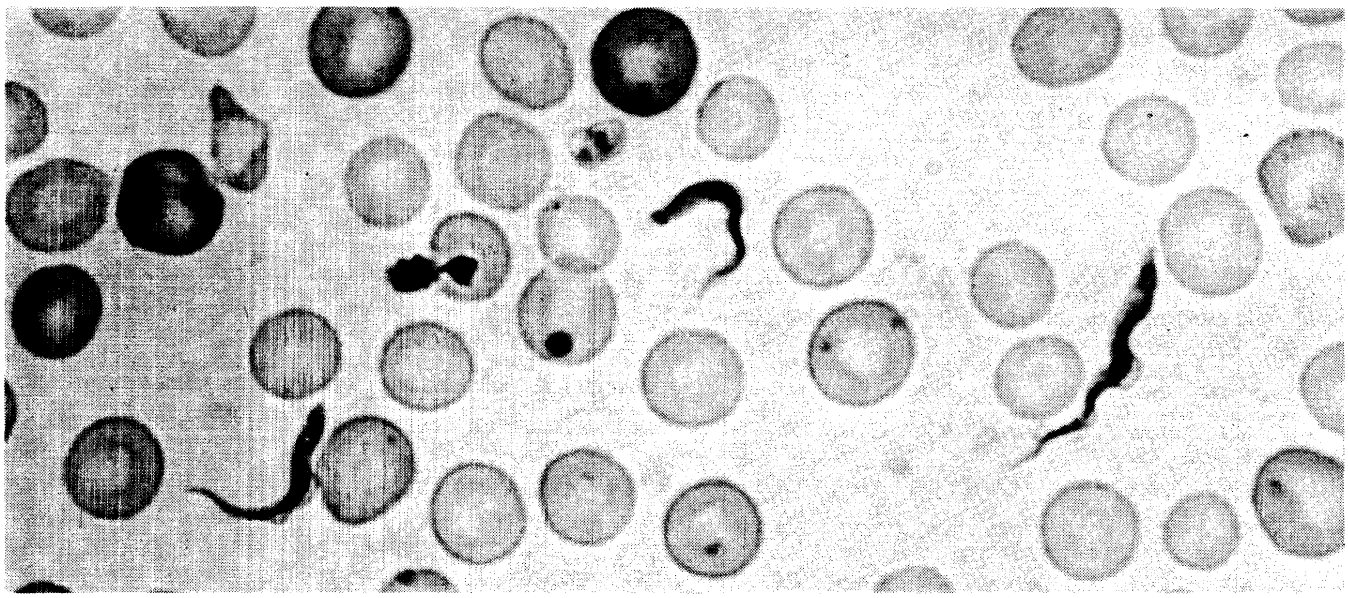

Figure 9. Three different morphological types of bloodstream *Trypanosoma congolense:* **intermediate (left), short (middle) and long (right). 940×.**

TABLE 6. The infectivity of individual organisms of a clone of *T. congolense*, related to their morphology

Morphological types	No. tested	No. infective*
Long forms	19	3
Intermediate forms	6	3
Short forms	19	15

*Tested using the χ^2 test of association; differences observed between 1 and 2 ($p<0.5$), 1 and 3 ($p<0.001$).

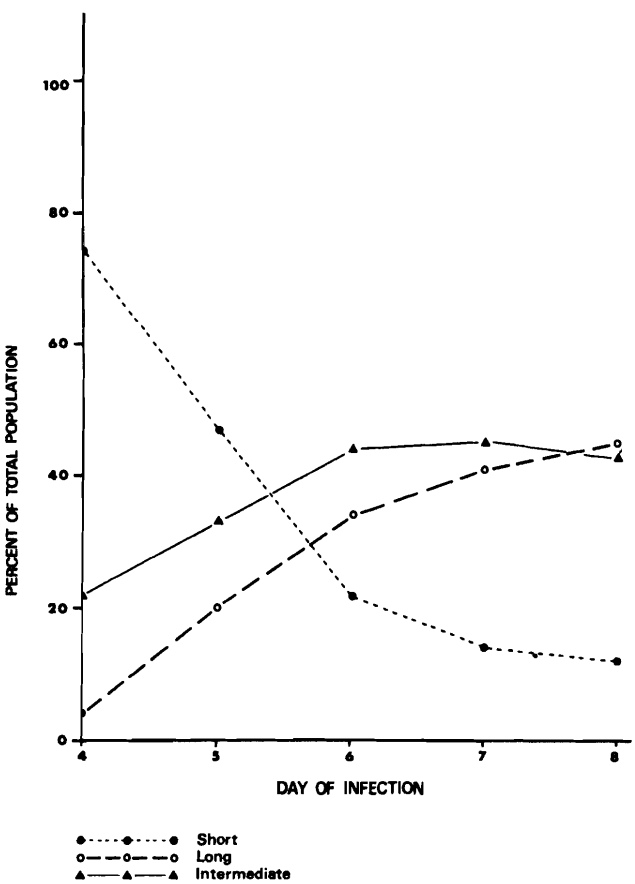

Figure 10. The morphology of *Trypanosoma* (*Nannomonas*) *congolense* **related to parasitaemia in the mouse.**

climatic conditions, were fed on mice whose parasitaemia had just reached its peak.

The infection rates fell significantly when the flies were fed on mice with rising or falling parasitaemias. The cycle of development in the fly varied from 7 to 40 days with 45% of the infections becoming patent after 12 days and 76% after 18 days. Thus the developmental cycle of *T. congolense* in *G. morsitans* is apparently shorter than the 19–53 days previously reported. Infected flies were maintained under standard conditions for as long as 150 days without loss of their infections. The development of such standard conditions and techniques made it feasible to carry out the experiments requiring antigenic analyses of metacyclic trypanosomes of *T. congolense*, described elsewhere in this report.

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STUDIES ON MOUSE-INFECTIVE *TRYPANOSOMA VIVAX*

In the ILRAD report of 1976 the importance of *T. vivax* was stressed and a description given of the strains under investigation.

From experiments done during 1977 it became clear that although the use of a mouse model has some advantages, one should be very careful in drawing conclusions relevant to the natural host. An intensive study has been made of the physiology and morphology of the parasite. These studies will be continued.

Unlike *T. congolense* and *T. brucei*, bloodstream populations of *T. vivax* do not undergo cyclic transformation in length and infectivity during each parasitaemic wave. Moreover, variation in length is less in cloned populations than in the original uncloned populations. Mean length may vary between 20 μm and 22 μm. The length distribution of cloned populations is 18–25 μm and of uncloned populations 13–27 μm. Different morphological types present in the bloodstream may, however, differ in their individual ability to infect mammals or the tsetse fly as in *T. brucei* and *T. congolense*. This is at present being investigated.

While relatively much is now known about antigenic variation in *T. brucei* and *T. congolense*, some controversy still exists about the nature of antigenic variation in *T. vivax*. It was reported that the rat-adapted Desowitz strain absorbed large amounts of host serum proteins on its surface. Speculations have been made by several investigators about the function of this phenomenon, either in adaptation to a new host, as an additional mechanism to evade the host immune response, or in the production of variable antigens. We found it essential therefore to re-investigate this possibility with our mouse-infective strains. We were not able to detect mouse proteins on the surface of washed trypanosomes grown in mice using the following techniques: agglutination, cytotoxicity test, double diffusion, immunoelectrophoresis, indirect immunofluorescence and lactoperoxidase catalysed surface radioiodination of live parasites followed by polyacrylamide gel electrophoresis (page-SDS) and immunoprecipitation. Moreover, such parasites could be incubated for 1½ hours in anti whole mouse serum without losing their infectivity and virulence for mice. We propose therefore that the adsorption of host proteins is not necessary to produce infections of *T. vivax* in mice, and that it does not play a major role during the life of the parasite in the vertebrate host.

To be able to investigate antigenic variation it was necessary to produce populations from a single trypanosome—a clone. This appeared to be extremely difficult in mice. After many unsuccessful attempts only one clone was established in normal mice. All attempts in lethally irradiated mice were unsuccessful and this is an important limitation of the mouse model. Five clones were, however, easily established in goats.

Our mouse-derived clone has been used to investigate antigenic variations in goats and mice. The preliminary conclusions from results of immunolysis tests are as follows.

One single trypanosome has the genetic background to produce a large number of different antigenic populations in both mice and goats. Parasites with different surface antigens are present at the same time in the bloodstream. Antigenic variation in goats is a continuous process and the composition of the populations changes every one or two days. Predominant antigenic types exist but may be different in goats and irradiated and normal mice.

The first antibodies detected in the two goats used after fly transmission were directed against the same population which differed from the population ingested by the flies. These antibodies were also directed against the first relapse of the clone in both mice and goats. After tsetse transmission, antibodies against the population ingested by the fly occur late during infection.

The clones produced in goats will be used to investigate the presence of a basic antigen after fly transmission. For further detailed investigations variant specific immunofluorescence will be used.

The main advantage of the *T. vivax* mouse model is apparently the possibility to produce large amounts of trypanosomes for biochemical analysis, and to investigate the sequence of antigenic variants in mice. The main limitations are the difficulty in producing antigenically stable clones, and the not always identical composition of populations isolated in mice, both lethally irradiated and normal, as compared to the original populations in the ruminants.

Financial support for this work was provided by the Netherlands Foundation for the Advancement of Tropical Research.

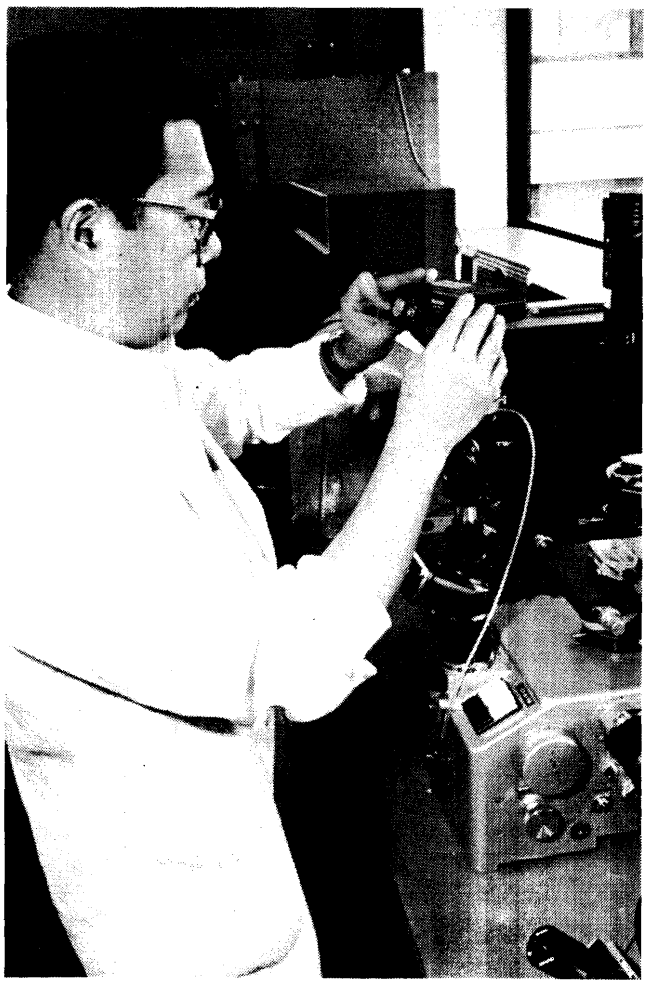
A. L. W. DE GEE

ANTIGENIC VARIATION IN THE SALIVARIAN TRYPANOSOMES: *IN VITRO* STUDIES

An understanding of the genetic basis, and possible inductive mechanisms, underlying the phenomenon of antigenic variation is important, in that this process represents a major avenue by which the trypanosomes establish a chronic infection in the face of protective immune responses by the host.

Prior to the development of *in vitro* culture techniques for bloodstream salivarian trypanosomes, it was impossible to devise controlled conditions to investigate this phenomenon as work had to be carried out in intact or immunosuppressed mice. The ability to continually cultivate bloodstream trypanosomes *in vitro* has now allowed us to investigate the question of variant antigen expression and stability of expression under defined conditions.

The results to date can be summarized as follows.



Dr. Hirumi preparing for microphotography of cultured trypanosomes.

A clone (type 52) of 427 *T. brucei* bloodstream trypanosomes was adapted to culture conditions using an inoculum of only 500 trypanosomes per culture. Fourteen days later these trypanosomes were recloned *in vitro* and five such clones selected for study. The antigenic type of trypanosomes in each culture was monitored by immunofluorescence at least weekly and 1000 trypanosomes scanned on each occasion. The original culture was also monitored in a similar fashion.

This original culture and its derivates remained antigenically stable (type 52) for 35 days of cultivation. Between days 35 and 43, however, a new antigenic type, 221, appeared and by day 50, when the experiment was terminated, this type had completely superseded the original (52) antigenic type.

In the case of the five recloned populations derived *in vitro*, two such populations changed their antigenic type from 52 to 221 after 20–30 days in culture, at which point the experiment was terminated. *In vivo* trypanosomes carrying antigenic type 221 consistently appear in the first relapse population of normal mice infected with clone 52 type trypanosomes.

These results indicate that previous suggestions, that host antibodies were solely responsible for the induction of antigenic variation, are not valid and that the phenomenon could be induced as in other protozoa such as paramecium and tetrahynena, by physiological stimuli such as glucose concentration, CO_2, etc. Host antibodies could also contribute to this stimulus and the role of various possible stimuli are now being investigated. The observation that one antigenic type (52) consistently changes to another (221) would support the hypothesis that the major genetic basis for this phenomenon is that of individual genes coding for different variant antigen molecules.

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ANTIGENIC VARIATION IN THE SALIVARIAN TRYPANOSOMES: THE EFFECTS OF CYCLICAL TRANSMISSION ON *TRYPANOSOMA (NANNOMONAS) CONGOLENSE*

While the tsetse fly has an obvious role to play in the epidemiology of trypanosomiasis by virtue of its ability to transmit the parasite from one host to the next, it also has a less well recognized role to play in the phenomenon of antigenic variation. This latter role is of utmost importance to our understanding of the immunology/epidemiology of this disease.

Over the last decade, evidence has accumulated in the case of *T. brucei* that if bloodstream trypanosomes had the same genetic constitution, but carried different surface variant antigens at the time of ingestion by the flies, the subsequent metacyclic stages transmitted by such flies all carried the same antigenic type. In view of the large number of variant types that can exist in a host or an area, this could be an important limiting factor in the number of types with which one would be concerned in deriving immunological control methods based on surface variant antigens.

It was therefore decided to investigate the existence of a similar phenomenon in the case of *T. congolense*. This work was rendered technically difficult by the fact that *T. congolense*–infected tsetse flies produce only a small number (20–50) of metacyclics at each feed, as compared to the 3000–4000 produced by *T. brucei*–infected flies. The techniques of immunofluorescence and neutralization used in the antigenic analyses of such metacyclic populations had therefore to be scaled down to cope with such small numbers of parasites, a technical problem which has been successfully overcome. The results of these experiments are summarized as follows.

When genetically related, but antigenically distinct, bloodstream populations of recently isolated *T. congolense* were cyclically passaged through the tsetse fly and the metacyclics analysed for variant type, it was found that as in *T. brucei* such variants all reverted to a common metacyclic antigenic type.

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TABLE 7. Development of immunity to a cyclically transmitted clone of *Trypanosoma (Nannomonas) congolense*

Group	No. mice	Immunization schedule (no. infective fly bites on each mouse)		Challenge (no. fly bites on each mouse)	No. mice protected (parasite-free after challenge)
		First dose	Second dose		
I	7	5	.....	1	0/7
II	8	10–12	.....	1–2	0/8
Controls*	6	.....	.....	1	0/6
III†	10	12–14	12–15	2	10/10
Controls*	4	.....	.....	2	0/4
IV‡	6	.....	.....	1–2	0/6

*The Berenil controls received the drug without prior exposure to tsetse fly bites.
†This group of mice was subsequently protected against challenge by flies infected with antigenically different bloodstream derivatives of this strain.
‡This group of mice was immunized using a bloodstream trypanosome population isolated from a fly-infected mouse.

To ensure that such variant populations were genetically related, a series of such populations was derived initially from the descendants of a single trypanosome.

If flies were allowed to feed on a host carrying a mixture of variant types of different genetic background, then the metacyclic types subsequently reflected this genetic heterogeneity, with each metacyclic antigen specific for a given genetic type appearing simultaneously, i.e. a fly can transmit more than one metacyclic antigenic type if the ingested population is genetically heterogenous.

These results would indicate, therefore, that in any given area, even though a large number of variant types exist in infected animals, the number of variant types transmitted by the tsetse will be limited to the number of different genetic backgrounds that exist in the area. If this is limited, then the number of different metacyclic types will also be limited. It must however be borne in mind that other biting flies as well as tsetse may transmit variant types directly by mechanical rather than cyclical transmission, and the implications of this form of transmission on the epidemiology of the disease have still to be elucidated.

In view of this inherent limitation of antigenic challenge imposed by cyclical transmission, it becomes important to know if animals can develop a successful immunity to such infections. This was investigated by exposing mice to various regimes of bites from infected flies transmitting variants of one strain of *T. congolense*. Initially these infections became patent and the mice were treated to remove the infection as were the unbitten controls. After a total of 24–29 bites such mice developed a complete immunity to challenge with such flies, compared to treated but uninfected controls, but were susceptible to challenge with metacyclics of a genetically unrelated *T. congolense* population. The results are summarized in table 7. This is an important observation, in that it could indicate that in the field, animals can become immunized to the spectrum of metacyclic antigen which exists in their area over some period of time. This is one way in which the increasing resistance of cattle with age, observed in trypanosome-endemic areas, could develop. Such experiments are to be repeated in cattle to investigate the existence of similar protective immunity.

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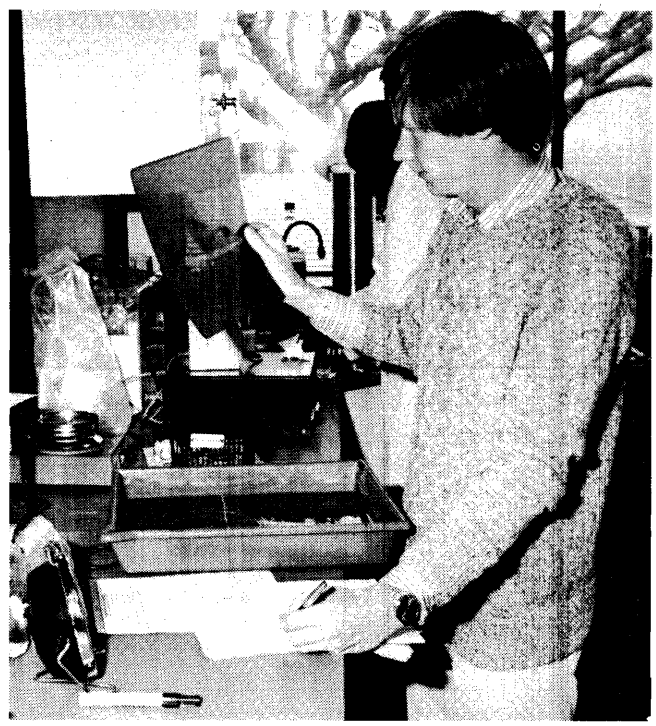
CROSS-REACTING DETERMINANTS IN VARIANT SPECIFIC SURFACE ANTIGENS OF AFRICAN TRYPANOSOMES

The problems of developing a useful vaccine against African trypanosomes centre on the ability of the organisms to undergo antigenic variation. Antigenically distinct types have been observed by the use



Dr. A. Barbet examining a peptide map of trypanosome variant antigens.

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of the antibody agglutination reaction at intervals during the course of an infection. It is known that antibody against a single variant type of trypanosome can agglutinate that variant, but not other variants arising from the original infecting population. The ability of trypanosomes to express variant antigens is associated with the presence of a surface structure—the coat, a layer 12–15 nm thick, overlaying the cytoplasmic membrane.

Recently, using the N-acylating reagent formylmethionine-sulphone methyl-phosphate (FMSMP) as a surface label, a glycoprotein, which is the major constituent of the surface coat, was successfully isolated by Cross from each of a number of clones of *T. brucei*. The glycoproteins from different variant clones had the same apparent molecular weight, about 65,000 as determined by sodium dodecyl sulphate gel electrophoresis, but could be distinguished by their isoelectric points. An antiserum against a purified surface glycoprotein stained by immunofluorescence the surface of trypanosomes of the clone from which the glycoprotein was derived, but not other trypanosome clones. Immunization of mice with purified surface glycoprotein protected against challenge with a small number of homologous, but not heterologous, trypanosomes. No immunological cross-reactions between the purified antigens were observed; neither were there any similarities in the extreme N-terminal amino acid sequences of a small number of surface glycoproteins. A second surface labelling technique, ^{125}I-iodination catalysed by lactoperoxidase, has been used at ILRAD in characterization of the surface coat of *T. congolense*. The variant specific surface antigen (VSSA) of *T. congolense* has a slightly smaller apparent molecular weight than that of *T. brucei*.

In some respects it is surprising that immunological cross-reactivity has not previously been observed between VSSAs. Although the data show that VSSA regions exposed on living trypanosomes are antigenically distinct, this is not necessarily the case with non-exposed parts of the molecule. Arguments for some conservation of structure between different VSSAs seem reasonable. For example, there might be a common region of the molecule responsible for membrane binding. The surface coat is the initial barrier between the organisms and the external environment; therefore specific permeability properties may be required, which could depend on some conservation of the three-dimensional structure. Our experiments show that antigenic similarities do exist between isolated VSSAs of trypanosomes.

Seven variant specific surface antigens were purified from clones of *T. brucei* and *T. congolense* and tested for immunological cross-reactivity. Anti-VSSA sera were clone specific when tested by indirect immunofluorescence of living trypanosomes, but they were not clone specific when tested by radioimmunoassay with purified ^{125}I-labelled VSSAs. In this double-antibody radioimmunoassay every VSSA tested was precipitated by the homologous and all heterologous anti-VSSA sera (fig. 11). Any unlabelled VSSA could inhibit the heterologous precipitation reactions by 100%. The homologous precipitation reactions were effectively inhibited only by unlabelled homologous VSSA (table 8). Cross-reactions between different VSSA molecules were also shown by micro-complement-fixation tests. The experiments confirm the presence in VSSAs of variable determinants specific to individual VSSAs and also show cross-reacting determinants in all VSSAs tested, including those isolated from different species of trypanosome. These results contrast with previous studies of other workers which failed to find evidence for immunological cross-reactivity between different VSSA molecules.

If cross-reacting determinants in VSSAs represent similar structural regions, as seems likely, questions

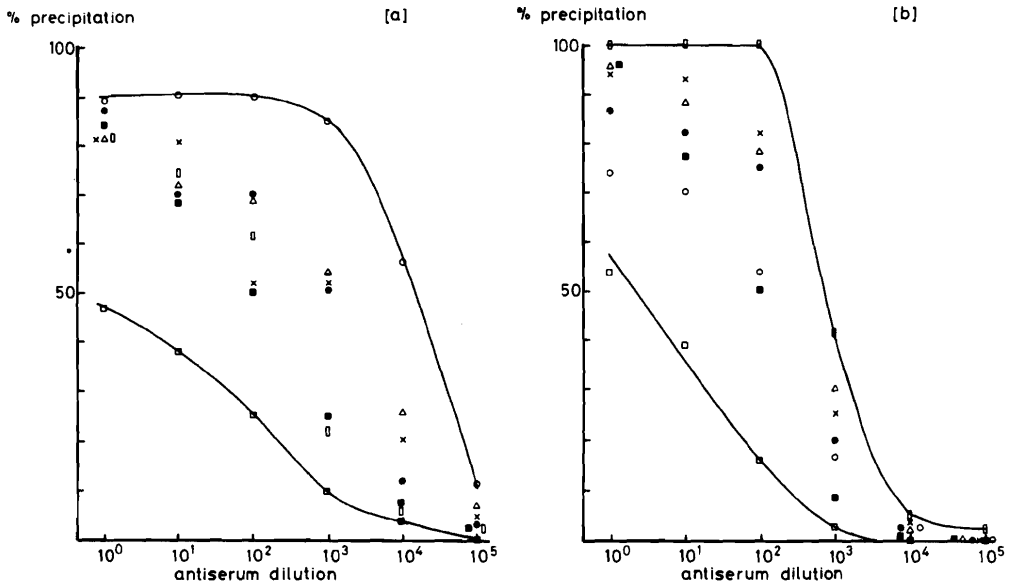

Figure 11. Double-antibody radioimmunoassay plots for ^{125}I-049 VSSA (a), ^{125}I-367 VSSA (b) and anti-VSSA sera; anti-367 (□), anti-9C (■), anti A4 (×), anti-049 (o), anti-052 (●), anti-055 (Δ) and anti-*T. congolense* X4 (□).

TABLE 8. Relationship between cross-reacting determinants of various VSSAs

Antibody	ng VSSA required for 50% inhibition					
	367	9C	A4	055	052	049
Anti-367	6	0%*	0%*	14%*	18%*	0%*
Anti-052	5	12	9	3	15	5
Anti-X4	12	4	10	16	16	7

Each assay tube contained 4 ng of ^{125}I-labelled 367 VSSA. Antisera dilutions were selected to give 50% precipitation of labelled antigen.

*Where 50% inhibition was not achieved the % inhibition obtained using 800 ng VSSA is shown.

arise concerning the extent and intramolecular location of such regions. One possibility for the location of cross-reacting determinants in VSSAs is a membrane-binding sequence, perhaps including sugars. A second possibility has been suggested by our results, showing that many VSSAs are susceptible to endogenous proteolytic degradation, producing a series of fragments each differing in molecular weight by approximately 8000 daltons. It is tempting to speculate that there are a number of similar sites distributed at intervals throughout each VSSA molecule, susceptible to proteolytic cleavage, and representing the cross-reacting determinants.

We are continuing our investigations by performing detailed structural analyses on VSSA fragments containing cross-reacting determinants. We hope that these studies will reveal the extent of structural homology between different VSSAs and provide insight into the molecular changes occurring during the process of antigenic variation.

A. F. BARBET
T. C. MCGUIRE

SURFACE LABELLING OF *TRYPANOSOMA VIVAX*

Mouse-infective *T. vivax* are surface labelled *in vivo* by an enzymatic iodination procedure utilizing lactoperoxidase, I-125 and hydrogen peroxide generated by a glucose oxidase-glucose system. All incorporated isotope is on the surface of the parasites and the procedure resulted in no significant cell lysis. I-125–labelled membrane proteins are solubilized by 0.5% NP40, separated from insoluble material by ultracentrifugation, dialysed and analysed by sodium dodecyl sulphate polycrylamide gel electrophoresis (SDS-PAGE) and immunoprecipitation with the following antisera: rabbit antitotal *T. vivax* which was obtained by immunizing with whole parasites and was shown to be specific for virtually only one parasite antigen by immunoelectrophoresis and immunodiffusion analyses; rabbit anti–*T. brucei* variant specific surface antigen (VSSA) 055; several antitotal mouse serum proteins; and normal rabbit serum.

SDS-PAGE of total labelled parasites and of NP40 soluble material show the same profile upon radiography of gels. The majority of labelled surface molecules are concentrated into a broad band of proteins ranging between 52,000 and 57,000 daltons molecular weight. Such a broad band could be resolved into two to four bands in different experiments. These results suggest that the surface coat of *T. vivax* is composed either of a restricted protein population with slightly different molecular weights or of a single species (as *T. brucei* and *T. congolense*) whose degradation products are responsible for the multiple-band SDS-PAGE profile.

Direct immunoprecipitations were carried out with the antisera listed above and with I-125–labelled NP40 soluble supernatants. Autoradiography of SDS-PAGE of washed precipitates showed that the rabbit antitotal *T. vivax* antiserum recognizes the

George Mpimbaza and Phelix Majiwa performing gel electrophoresis of variant antigen made *in vitro*.

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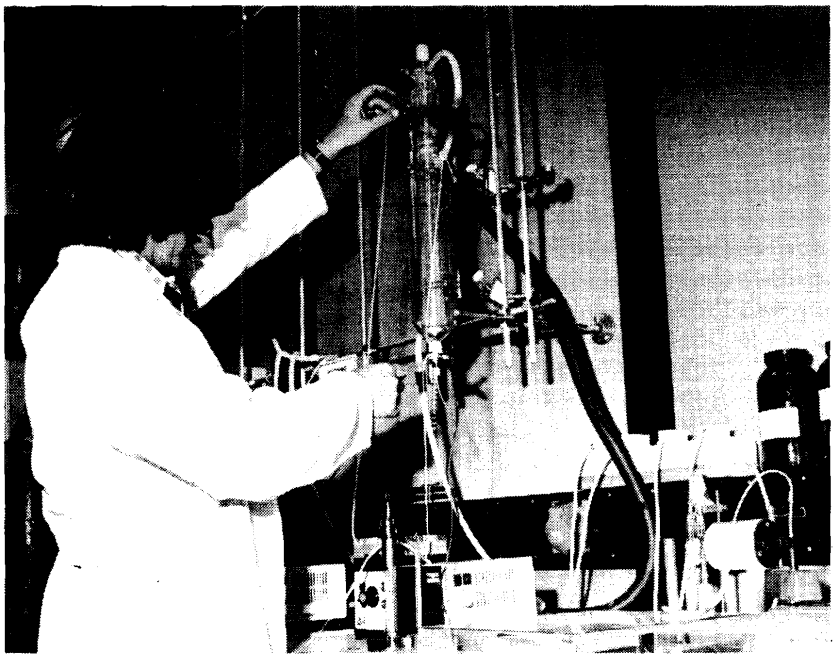

Research assistant Johanna Vernooij preparing an isoelectric focusing column.

same components detected by SDS-PAGE analyses of total labelled parasites, thus suggesting that the antiserum specificity is directed against the major surface antigen(s) and not against other cell components. When tested in the same system, a rabbit antiserum specific for *T. brucei* clone 055 is also precipitating the same proteins, although to a lesser extent. Normal rabbit serum used as a control, and rabbit antitotal mouse proteins antiserum, did not precipitate any specific radioactivity. These preliminary results suggest that (*a*) the surface coat of *T. vivax* has an overall structure similar to *T. brucei* and *T. congolense*, (*b*) the main surface component(s) cross-react with immunogenic groups present in *T. brucei* VSSA, and (*c*) host serum proteins are not present on the surface of the parasites.

A. L. W. de Gee
L. Rovis
J. Vernooij

PURIFICATION AND CHARACTERIZATION OF *TRYPANOSOMA BRUCEI* PLASMA MEMBRANES

T. brucei cloned bloodstream forms are disrupted by nitrogen cavitation in isotonic sucrose. Cell components are fractionated by differential centrifugation at 450 *g*, 4500 *g*, 10,000 *g*, 100,000 *g*. The various pellets are comparable to conventional nuclear, mitochondrial kinetoplast, and microsomal fractions; however the degree of purity of each organelle in such fractions is questionable. Morphological criteria of evaluation are not satisfactory and so far only a few enzyme markers have been identified. The 100,000 *g* supernatant contains only soluble materials comprising more than 50% of total *Trypanosoma* proteins. Approximately 30% of the soluble protein is variant surface specific antigen (VSSA). Total cell homogenate, the four pellets and the soluble fraction are tested for protein, lipid, DNA and RNA contents and for the following enzymatic activities: Na^+, K^+, Mg^{2+} ATPase, 5'-nucleotidase, glucose-6-phosphatase, acid phosphatase and glycerol-3-phosphate dehydrogenase oxidase.

Plasma membranes are separated from the microsomal fraction (100,000 *g* pellet) by high-speed centrifugation on Dextran 170 and further purified by linear sucrose density gradient. The purified plasma membrane has a density of 1.14–1.15 g/cm³ and contains about 3% of total *Trypanosoma* insoluble proteins. Lipid content is 0.552 mg/protein. Cholesterol/phospholipid molar ratio is 0.54. Membrane-bound surface variant antigen has been qualitatively detected by immunodiffusion using NP40 solubilized membranes and a rabbit antiserum specific for the VSSA of the same clone used for membrane preparation. Of several enzymes tested

Dr. S. Baekkeskov working on the isolation of lipids from African *Trypanosoma*.

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only Na^+, K^+, ATPase seems to be a reliable plasma membrane marker and consistently shows a severalfold increase of specific activity over the total cell homogenate. 5'-nucleotidase is also present but is not a plasma membrane marker.

Further chemical characterization and electron microscopy are necessary to assess the purity of our preparations before we start to study the immunological properties of plasma membranes and the role that they might play in the pathogenesis of trypanosomiasis.

L. Rovis
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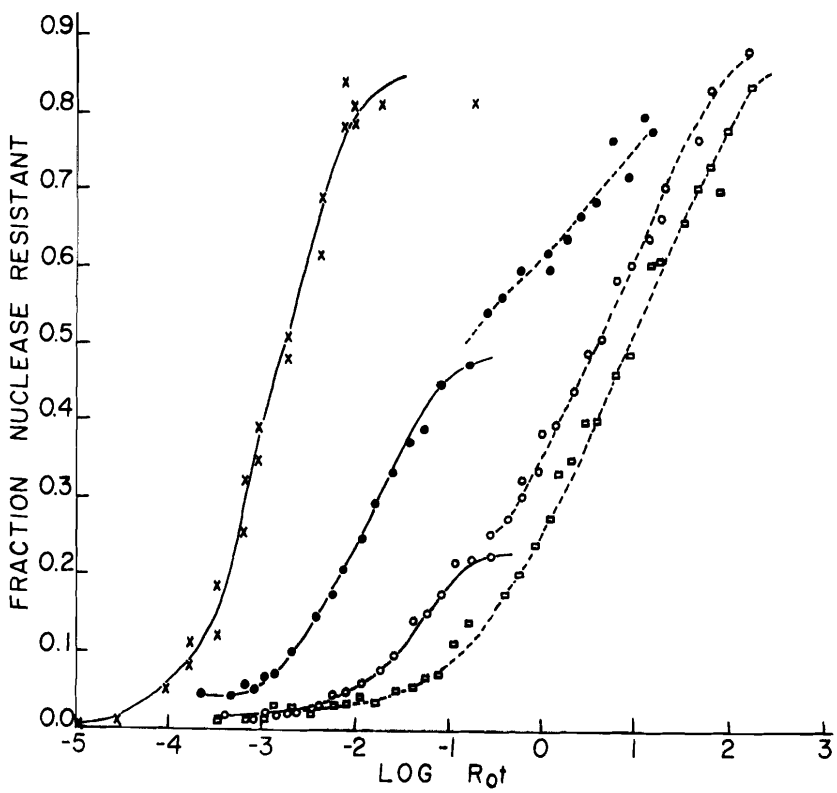
J. Vernooij

CHARACTERIZATION OF *TRYPANOSOMA BRUCEI* POLYADENYLATED RNA

The major parasitic African trypanosomes, aetiological agents for human sleeping sickness and nagana in cattle, have evolved intricate mechanisms for maintaining a chronic pathogenic infection by evading or limiting the immune capabilities of the host. It is believed that one of the mechanisms contributing to this chronic infection is the ability of the parasites to alter their membrane antigens. It has been shown that the variant specific surface antigen can be used successfully as a vaccine when the host is challenged with the homologous cell clone of trypanosomes. It remains vital to the development of a vaccine to know the capacity of the trypanosome to vary its antigenic structure. In this context it is important to know the number of potential variants that a single cell can express and the mechanism by which variation occurs. The presence of an abundant mRNA in trypanosomes coding for the variant antigen would facilitate the work towards understanding the molecular processes involved.

Polyadenylated RNA was isolated from *T. brucei* and was shown to direct the synthesis of the variant antigen protein in an *in vitro* system. The trypanosome mRNA was electrophoresed under denaturing conditions and RNA between 1.9 and 2.5 kb was eluted from the gels and shown to direct the synthesis of the variant antigen. The variant antigen message is approximately 2 kb. The complexity of both total cellular mRNA and mRNA between 1.9 and 2.5 kb was measured. Of the total mRNA, 20% consists



Hybridization of

a) Kappa 321 mRNA to kappa 321 cDNA (×–×), $R_0t\frac{1}{2} = 1 \times 10^{-3}$ mol. sec-litres^{-1}

b) mRNA eluted from a formamide gel, 1.9 to 2.5 kb, to its homologous cDNA probe (●–●). Calculating from the plotted theoretical curve, the transition seen at 47% hybridization gives a corrected $R_0t\frac{1}{2}$ of 6×10^{-3}. This indicates that 42% of the RNA in this size class has a complexity of 7.2 kb. This would be the equivalent of 2 to 3 messengers each between 1.6 and 2.4 kb.

c) Total trypanosome (clone 11E1) mRNA to its homologous cDNA probe (o–o). The plotted theoretical curve yields a corrected $R_0t\frac{1}{2}$ of 8×10^{-3} indicating that 20% of the trypanosome RNA has a complexity of η^{11} kb.

d) Total trypanosome (clone 3E9) mRNA to cDNA from clone 11E1 (□–□). The abundant RNA sequences seen in hybridization c are absent indicating that much of the low complexity RNA is clone specific.

Figure 12. Complexity analysis of polyadenylated RNA. Radioactive cDNA probes were synthesized using AMV reverse transcriptase from (1) total cytoplasmic polyadenylated RNA and (2) RNA, 1.9 to 2.5 kb in size (fraction 3 above), eluted from the formamide gel. The hybridizations were measured by S_1 nuclease resistance. As a kinetic standard a cDNA probe was synthesized from highly purified mRNA coding for kappa 321 immunoglobulin light chain. Hybridization of this probe with homologous driver (1.2 kb) gave a $R_0t\frac{1}{2}$ of 1×10^{-3} mol. sec-litres^{-1}. This value was used in the analysis below. Theoretical curves were constructed for a best fit of the data and are illustrated by solid lines.

of 3 to 4 mRNA sequences (11 kb), and 42% of the mRNA 1.9 to 2.5 kb consists of 2 to 3 mRNA sequences (7.2 kb). (See fig. 12.) These data indicate that the variant antigen mRNA is an abundant RNA sequence in the trypanosome cytoplasm. Studies are under way to clone the variant antigen genes in bacteria in order to study the mechanism of antigen variation and to reveal the antigen gene number.

R. O. WILLIAMS

CHARACTERIZATION OF DNA POLYMERASE FROM *TRYPANOSOMA BRUCEI*

Up to now the only evidence suggesting a possible mechanism of antigenic variation has been the amino acid sequence data of Bridgen et al.* Because only a very small portion of the variant antigen protein was sequenced, it would be difficult to make conclusions on the mechanism of antigenic variation from these data. Very recent work showing cross-reacting antigenic determinants of the variant antigen proteins would suggest that there may be alternative explanations for the great variability of the surface antigens, such as somatic rearrangement of genes and even possibly a mutagenic DNA polymerase. These possible explanations have not as yet been considered in the literature and deserve further attention.

Multiple species of DNA polymerases have been described in eucaryotic cells. In the case of mammalian cells, there is widespread agreement that three different DNA polymerases can be distinguished, in addition to mitochondrial DNA polymerase. These have been designated as DNA polymerase-α, -β, -γ, and *mt* (mitochondrial). However, recent evidence suggests that the DNA polymerase *mt* is a γ-polymerase. It seems likely that this classification scheme may eventually be applicable to the DNA polymerases present in most eucaryotic cells. DNA polymerase-β, the lower molecular weight and comparatively N-ethylmaleimide–resistant species of DNA-dependent DNA polymerase, has not been found in unicellular eucaryotes or plants.

The predominant DNA polymerase activity has been isolated from the parasitic flagellated protozoa, *T. brucei*. Like mammalian DNA polymerase-α, the trypanosome DNA polymerase is of large molecular weight (6S–8S), is resistant to thermal denaturation, is sensitive to N-ethylmaleimide, and is inhibited by high ionic strength. However, specific antisera that cross-react with mammalian DNA polymerase-α from different species fail to cross-react with the trypanosome polymerase.

R. O. WILLIAMS

*Bridgen, M., Cross, G.A.M., and Bridgen, P. 1976. N-terminal amino acid sequences of variant-specific antigens from *T. brucei*. *Nature* 263:613–614.

SURFACE ANTIGENS OF TRYPANOSOMES: STUDIES USING MONOCLONAL ANTIBODIES

Trypanosome variant specific surface antigens (VSSAs) are often studied using antisera raised in animals by injection of cloned populations of trypanosomes. Most antisera obtained in this way are complex mixtures containing antibodies of different specificities, affinities and classes and because of this complexity are inadequate for studying many of the problems associated with antigenic variation. For example, if the parasite population used for immunization contains a small number of trypanosomes of a different antigenic type, then the antiserum produced will almost surely contain antibodies to these variants. Such an antiserum will detect all of the antigenic types present in a mixture, leading one to believe that it is homogeneous (with respect to antigenic type).

Dr. T. W. Pearson and Dr. G. E. Roelants working with the fluorescence-activated cell sorter, the first of its kind in Africa.

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The use of monoclonal antibodies avoids this kind of problem and in addition allows more defined experimental approaches and facilitates experiments difficult or impossible with conventional antisera such as:

1) Determination of the number of antigenic variants arising in trypanosome populations (fluorescent monoclonal antibodies in combination with the fluorescence-activated cell sorter).
2) Production of specific high-titre antibodies to 'common region' determinants on VSSAs.
3) Isolation of pure VSSAs by immunoabsorbant techniques.
4) Immunoprecipitation of antigenic products from cell-free protein synthesizing systems. High-titre monoclonal antibodies (monospecific?) are ideal for genetic engineering (recombinant DNA) technology involving the VSSAs.

Monoclonal antibodies to trypanosome VSSAs will enable standardized assays for a single variant type to be distributed internationally. Identification of antigenic types and comparison of results between laboratories will thus be facilitated.

At ILRAD, we have immunized mice and rabbits with VSSA from *T. brucei*, have performed two fusions, and are isolating hybrid cells by growth in selective medium. Screening for antibody-producing hybrids using a radioimmunoassay has shown 21 fusion mixtures are producing antibody to purified VSSA from *T. brucei*. Cloning of the hybridomas in soft agar is now under way.

T. W. Pearson
T. C. McGuire
R. O. Williams
L. B. Lundin
J. Young

SUPPRESSION OF IMMUNE RESPONSIVENESS IN *TRYPANOSOMA CONGOLENSE*-INFECTED MICE

Trypanosome infection is known to depress immune functions. Many mechanisms may be involved. Last year we reported that there were profound changes in the spleen cells of infected mice and that polyclonal B cell activation could be instrumental in immune depression by leading to clonal exhaustion. This year we have focused on the extent and kinetics of depression and on the possible role of active suppressor cells.

Spleen cells from mice infected with *T. congolense* showed reduced lymphocyte reactivity as early as 7 days after infection. This immune depression paralleled the appearance of parasites in the peripheral blood and was generalized, since these spleen cells showed reduced lymphocyte stimulation in response to T and B lymphocyte mitogens and to histoincompatible cells differing at either H-2 or Mls loci. Mixed lymphocyte cultures (MLCs) containing these spleen cells, either as responders or as stimulators, also failed to generate cytotoxic lymphocytes. We showed that this depression was not simply due to the smaller proportion of T cells in spleen cell populations from infected mice, by using dilutions of normal T cells. These results suggested the presence of active supressor cells in infected mouse spleens. This was confirmed by showing that lymphocyte stimulation, induced in normal (uninfected) spleen cell populations by mitogens or by allogeneic cells, was suppressed by the addition of spleen cells from infected mice, whereas addition of spleen cells from uninfected control mice had no effect. In MLR the suppression was strongest when the suppressing cells and responding lymphocytes were syngeneic. Allogeneic skin grafting experiments, performed with normal and infected mice, showed that a depression of T lymphocyte activity also occurs *in vivo*. Moreover, the kinetics and degree of suppression studied in various mouse strains paralleled the degree of susceptibility as studied by Dr. Ivan W. Morrison.

Spleen cells from infected mice did not function as stimulators in MLR. Here, dilution analysis of normal B cells showed that reduced stimulation could merely be because of dilution of B cells by null cells. Moreover, 98% pure B cells from infected mice, obtained by sequential agglutination with soya bean agglutinin (SBA) and treatment with anti-Θ serum and complement, had intact stimulatory capacity in MLR. These results indicate that null cells might lack the relevant MLR stimulatory Ia antigens. This was confirmed by using anti-Ia antisera in cytotoxicity, absorption and immunofluorescence studies, using the microscope or the fluorescence activated cell sorter. On the other hand, null cells appeared to express 4 to 8 times more H-2 antigen than normal spleen lymphocytes.

The results suggest that the generalized immune depression, seen in mice infected with African trypanosomes, is due to suppressor cells. It is reasonable to believe that this suppression may be a major mechanism by which trypanosomes escape rejection from the host, in addition to and independent of antigenic variation.

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L. B. Lundin
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K. S. Mayor-Withey

TSETSE FLIES AS VECTORS OF TRYPANOSOMIASES

While building of the new tsetse unit was in progress, studies carried out last year were continued in the temporary facilities which were developed in two isolation units.

Previously *T.* (*Duttonella*) *vivax* (Zaria Y486) was cyclically transmitted by *G. morsitans morsitans* from infected to clean mice and also from infected to clean rabbits (ILRAD Research Report 1976). This work was carried further using rats and again without recourse to splenectomy or the administration of any supplementary bovid serum. In the first three groups of experiments using *G. m. morsitans*, attempts to cyclically transmit the infection between rats failed, but two out of the five rabbits challenged became infected. Infection rates in the vector varied from 0 to 10%. Parasitaemia in the rats, which were infected by syringe passage and used for infecting the flies, was invariably low. In the fourth attempt comprising three groups, the infection was initially subpassaged from rats to rats several times; the parasitaemia increased with subpassages. In this experiment *G. m. morsitans* and *G. m. centralis* were fed on rats showing high parasitaemia. These tsetses were eventually used to challenge four clean rats, three rabbits and a goat. The infection developed in three rats, all three rabbits and the goat; two rats and the goat died but the remaining animals aborted the infection. The mean infection rates in *G. m. morsitans* and *G. m. centralis* were respectively 18.2% and 29.4%.

The infection rates of the above *T.* (*D.*) *vivax* in *G. m. morsitans* and *G. m. centralis* were compared between the males and females, and between the teneral and non-teneral flies. There were no significant differences between them in terms of their infection rates. Also, there were no significant differences in the infection rates between the above two tsetse subspecies for both teneral and non-teneral flies. Teneral, 10-, 20- and 30-day-old *G. m. centralis* were fed on the *T.* (*D.*) *vivax*–infected goat. The subsequent infection rates in the flies were respectively 91%, 90%, 73% and 82%. This experiment is still in progress to collect additional data for statistical analysis, but the present preliminary results seem to suggest that there is little, if any, effect between age of the vector at the time of the infected blood intake in terms of the subsequent infection rate.

G. m. centralis were fed *in vivo* upon the *T.* (*D.*) *vivax*–infected goat or *in vitro*, using the silicone membrane feeding system, upon the infected defibrinated blood of the above goat. The mean infection rates were respectively 54% and 65%, but the difference was not significant at the 1% level.

The efficiency of the warm-slide probe has been compared with the standard dissection method for determining the mature *T.* (*D.*) *vivax* infection rate in *G. m. morsitans*. The flies were infected by feeding them *in vivo* on an infected goat. Whereas the former technique revealed mature infection rate of only 9.4%, the examination of hypopharynx of the flies that had probed showed trypanosomes in 67.7% of the vectors. The metatrypanosomes of *T.* (*D.*) *vivax* attach themselves anteriorly to the inner wall of the hypopharynx, and it is probable that they are not readily dislodged during probing on a warm slide. It is clear that for *T.* (*D.*) *vivax*, the warm-slide probe technique is highly inferior for determining mature infection in the vector compared with the standard dissection method. This experiment was repeated using *T.* (*Nannomonas*) *congolense* (×4). The mature infection rates in *G. m. centralis* using the warm-slide probe and dissection methods were respectively 15.0% and 22.6%. Again, the former method is inferior to the examination of hypopharynx of the *T.* (*N.*) *congolense*–infected flies, but the difference in the results was not as high as that for the *T.* (*D.*) *vivax*–infected vector.

Ten *G. m. morsitans* showing mature *T.* (*D.*) *vivax* (Zaria Y486) infection by the warm-slide probe method were fed individually on mice, about every 10 days. Only 2 out of the 55 mice used became infected and only one fly transmitted the infection to these 2 mice, one when challenged by this tsetse on day 93 and the other on day 104 after fly emergence. However, 2 different flies with the mature infection fed similarly on 20 clean goats, 10 in each case, transmitted the infection respectively to 6 and 5 goats. These 2 flies failed to transmit the infection to 2 mice when fed individually, but 2 flies used for the mouse challenge experiment described earlier that had failed to transmit successfully infected 2 goats. A group of 7 flies from the mouse-challenge experiment when fed on a single mouse also failed to transmit the infection to this host. It is thus concluded that cyclical transmission of this parasite to mice is far more difficult than to goats. Also, the *T.* (*D.*) *vivax* (Zaria Y486)–infected fly can transmit the infection to goats throughout its life.

A group of *G. m. centralis* was fed *in vitro* upon the defibrinated *T.* (*D.*) *vivax*–infected blood of a goat, showing the first rising parasitaemia when the number of parasites was about 10 per field of ×400 on a wet blood film (10/×400). Another group of flies was fed similarly but when the parasitaemia in the same goat was at its first peak (+40/×400). The subsequent infection rates in the two groups were respectively 62% and 78%. The difference is small but further work is in progress to determine whether such small difference is significant. In another experiment, the number of parasites in the defibrinated infected blood taken from the same goat, at the first rising (10/×400) and the first peak (+40/×400) parasitaemias, was in each case reduced by dilution with defibrinated clean blood of a goat to about 5 parasites/×400. Two groups of *G. m. centralis* were fed on these bloods as described earlier. Their subsequent infection rates were 57.9% and 60.5%, respectively. Further experiments involved *in vivo* feeding of four groups of *G. m. centralis* on the *T.* (*D.*) *vivax*–infected goat during the first four peaks of parasitaemias. The subsequent infection rates in the vector were respectively 100%, 100%, 97.1% and 85.3%. The differences are clearly low, but further work is in progress to ascertain if such

small differences are of any significance. The time of death of the infected goats after challenge was between 12 and 39 days (mean 23.6 days) for direct syringe passage, and between 16 and 82 days (mean 40.2 days) for cyclical transmission.

To our knowledge *T.* (*D.*) *vivax* isolated from East Africa cannot infect experimental rodents, but some West African strains of this trypansome species have been maintained in such hosts for experimental purposes. It was therefore of interest to screen some East African isolates in rodents. In a preliminary experiment, two isolates (V4 and V13) from Uganda and two (V1 and V2) from Kenya were tested. Four goats were infected by syringe passage with these isolates; the infection of V4 and V13 developed in goats while those of V1 and V2 failed to do so. The V4 and V13 infections in goats were successfully transmitted by syringe passages to mice, rats and rabbits, but all these hosts eventually aborted the infection. Further studies on V4 showed that it was possible to maintain this isolate in mice for at least 25 subpassages. The parasites in mice from this last subpassage have been cryopreserved in liquid nitrogen. Dissection of a large number of *G. m. morsitans* and *G. m. centralis* on about day 20 after the infected blood-meal intake from the V4 infected goats did not show any infection in these vectors. Hence, this *T.* (*D.*) *vivax* can be transmitted by syringe passage to mice, rats and rabbits from an infected goat, but cyclical transmission between goats using *G. m. morsitans* and *G. m. centralis* has so far failed. However, it was possible to transmit this isolate from an experimentally infected Zebu to a goat by mechanical transmission, using five interrupted feeds, and then to another goat using only one interrupted feed by the above vectors. All mice and rabbits used in the above experiments aborted the infection. In the Zebu the appearance of the parasites in the peripheral circulation was sporadic but all goats in which the infection developed after syringe passage or mechanical transmission died between days 29 and 132 after challenge (mean 55.8 days). No flies dissected between days 20 and 40 after blood-meal intake from the infected goats or Zebu showed any infection. It is possible that development of the parasite took place very early and was absorbed soon afterwards, or that the above two isolates have lost the characteristic cyclical development in the vectors. If the latter suggestion is correct, then it is conceivable that in the two areas of Teso District, Uganda, from where these parasites were isolated, transmission of the infection to different hosts is by mechanical means.

Teneral *G. m. morsitans*, fed *in vitro* upon the bloodstream forms of *T.* (*Trypanozoon*) *brucei* (427) in culture (see ILRAD Research Report 1976) and then fed on mice, rats and a rabbit on about day 35, failed to transmit the infection to these hosts. On about day 40 after the infected blood-meal intake, a group of flies were dissected; their salivary glands lacked infection, but their midgut infection rate was 2%. When the paired salivary glands of flies with midgut infection were individually injected into mice, no infection developed. However, tenerals of this insect similarly fed upon defibrinated blood of rats at the peak parasitaemia, which had been injected with the original isolate 427, transmitted the infection to a rabbit on which these flies were maintained, but the challenged rats remained negative. Dissection of a group of these flies showed no salivary gland infection but their midgut infection rate was 32%. Injection of the paired salivary glands of flies showing midgut infections resulted in 2 positive mice out of 16 used. The remaining two groups of flies, the one that had fed on the infected culture material and the other on the infected rat blood, were maintained for a further period on two rabbits and on about day 60 after the infected meal intake, the two groups were used to challenge rats. Both the rabbits and all rats remained negative. The midgut infection rates were respectively 4% and 5%, and injections of all paired salivary glands of flies, from both the groups with midgut infections, into individual mice gave negative results.

When the above flies were fed on the infected materials, the number of parasites in the defibrinated blood was $+40/\times 400$, while that in the culture was about $10/\times 400$. The parasitaemias of these infected materials at the time of fly feeding were at their peaks. Hence, in another experiment the infected rats were sacrificed when the parasitaemia was at its peak, and the number of parasites in the defibrinated infected blood was reduced to about $10/\times 400$ with defibrinated clean rats' blood. Teneral *G. m. morsitans* were fed as described earlier, maintained on a rabbit, and then on about day 40 a group of flies were fed on 3 rats and dissected on the following day. The midgut and proventricular infection rate was 9.1%. Three of the 5 infected flies showed parasites in the crop, and individual injection of mice with salivary glands from the above 5 infected flies, which showed no parasites by microscopic examination, gave negative result. Neither did infection develop in the 3 rats challenged. The remaining flies were maintained on the same rabbit, and on about day 60 were fed on 4 rats and then dissected. Four out of 46 flies dissected were found infected (infection rate, 8.7%): 1 with salivary glands, midgut and proventriculus, 2 with midgut and proventriculus, and 1 with midgut alone. Three flies showed parasites in the crop. The rabbit remained negative. One of the 4 rats became infected, and in 1 of the 4 mice injected with salivary glands the infection developed; this mouse eventually died. The infected mouse had received the glands with metatrypanosomes in them.

In addition to the above research activities, the tsetse unit carried out work in collaboration with other scientists using the three trypanosome species with the above vectors.

S. K. Moloo
S. B. Kutuza

EXPERIMENTAL IMMUNIZATION

Use of Immunostimulants to Increase Host Resistance

The problem of developing an effective vaccine against African trypanosomiasis is bedevilled by the ability of the trypanosome to undergo antigenic variation. As a result several other potential strategies are under consideration. One is the possible modulation of the host immune response to the trypanosome in order to produce a reaction more effective than normally occurs in the susceptible host where the response is characterized by a state of immunosuppression and hypergammaglobulinaemia levels, only a small proportion of which is directed against the trypanosome.

The powerful immunostimulants *C. parvum*, BCG (bacillus Calmette-Guérin) and *B. pertussis* were used to prime different strains of mice prior to challenge with *T. congolense* or *T. brucei*. The strains of mice used were A/J and SWR/J, strains susceptible to trypanosomiasis, and C57Bl/6J and C57Bl/10, strains reputed to be resistant. None of these immunostimulants prevented infection but all produced a significant increased duration of survival (tables 9–13). This was associated with marked changes in the parasitaemic profiles, in which the prepatent period and time to the first peak of parasitaemia were significantly lengthened, and in some cases there was a very significant reduction in the level of parasitaemia. This was specially marked when *B. pertussis* was used in A/J mouse challenge with *T. congolense* to the extent that the highly susceptible A/J strain mouse behaved in a manner more akin to the more resistant C57Bl/6J as judged by levels of parasitaemia and to some extent by survival (fig. 13).

TABLE 9. Effect of *C. parvum* on C57Bl/10 mice challenged with *T. brucei*

	C. parvum	Controls
Prepatent period in days	7.3*	5.1
Time to first peak of parasitaemia in days	10.0*	8.3
First peak of parasitaemia $\log_{10}$ trypanosomes/ml	7.66*	8.28
Time to death in days	32.0 *	21.4

*Significant to control

TABLE 10. Effect of *C. parvum* on SWR/J mice challenged with *T. brucei*

	C. parvum	Controls
Prepatent period in days	6.6*	5.5
Time to first peak of parasitaemia in days	10.3*	9.0
First peak of parasitaemia $\log_{10}$ trypanosomes/ml	7.85	7.73
Time to death in days	15.9*	12.5

*Significant to control

TABLE 11. Effect of *C. parvum* on A/J mice challenged with *T. brucei*

	C. parvum	Controls
Prepatent period in days	6.6*	4.5
Time to first peak of parasitaemia in days	10.5*	8.3
First peak of parasitaemia $\log_{10}$ trypanosomes/ml	8.02*	8.86
Time to death in days	15.4*	9.6

*Significant to control

Specific Vaccination Studies

In African trypanosomiasis protective immunity is generally regarded as being associated with the variant surface antigen which changes with each wave of parasitaemia. In the bovine, particularly the Ndama breed, the parasitaemic profile is characterized by progressive diminishing waves of parasites followed by elimination of the parasite in many cases. This might suggest the existence of an antigen, common between variants, which is involved in protective immunity. It might act by priming the host in some way for successive variants, i.e. producing a secondary or tertiary response to each variant.

In the present studies each trypanosome species is being subjected to critical subcellular fractionation and each fraction is being tested for protective ability in the hope that a common protective antigen does exist within and between species. It has been found that certain fractions such as flagella and the X fractions, as well as VSSA, are capable of stimulating complete protective immunity to homologous challenge (table 14). The X fraction is a mixture of kinetoplast and mitochondria. In both the X fraction and the flagella amounts of VSSA are also present.

TABLE 12. Effect of *C. parvum*, BCG and *B. pertussis* on mice challenged with *T. congolense*

		A/J control	A/J *C. parvum*	A/J BCG	A/J *B. pertussis*
Prepatent period in days		5.4	6.5*	6.7*	7.1*
Time to first peak of parasitaemia in days		9.8	11.4*	11.8*	10.9*
First peak of parasitaemia $\log_{10}$ trypanosomes/ml		8.41	8.60	8.50	8.08*
Percentage survival on day	10	68	100	96	96
	15	0	36	22	43
	20		23	18	43
	30		23	14	43
	40		18	14	39
	50		14	14	35
	60		6	4	30

* Significant to control

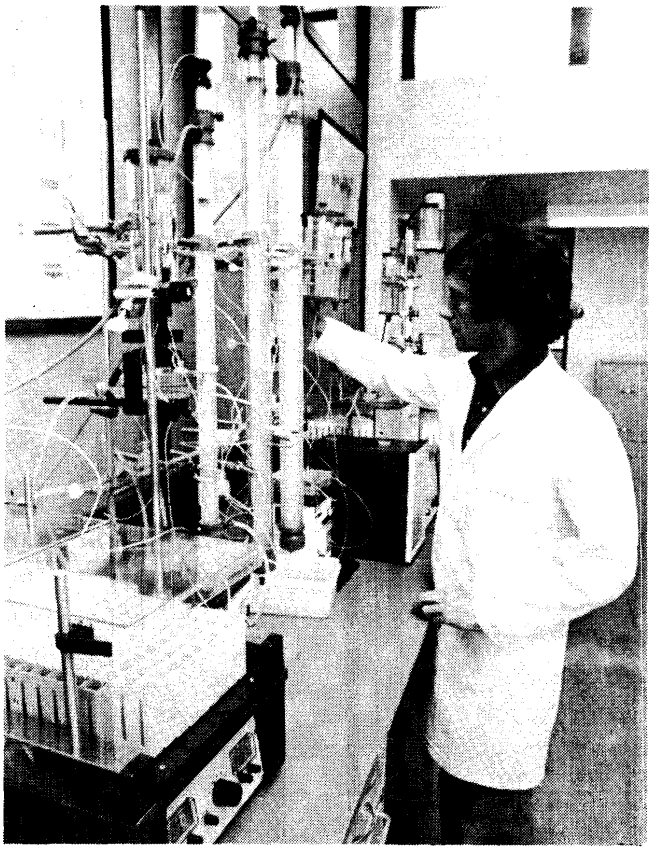
TABLE 13. Effect of *C. parvum* and BCG on C57Bl/6 mice challenged with *T. congolense*

	C. parvum	BCG	Control
Prepatent period in days	7.5*	5.3	5.2
Time to first peak of parasitaemia in days	11.5*	11.1*	9.8
First peak of parasitaemia $\log_{10}$ trypanosomes/ml	8.1	8.35	7.53
Time to death in days	114*	65	66

* Significant to control

Following heterologous challenge, i.e., challenge with another antigenic type, the X fractions produce a marked increase in survival time but no protective immunity. They might have acted as a non-specific immunostimulant the same way as *C. parvum*, BCG, or *B. pertussis*.

M. MURRAY
W. I. MORRISON
L. ROVIS



Dr. Ivan Morrison working on the separation of serum proteins.

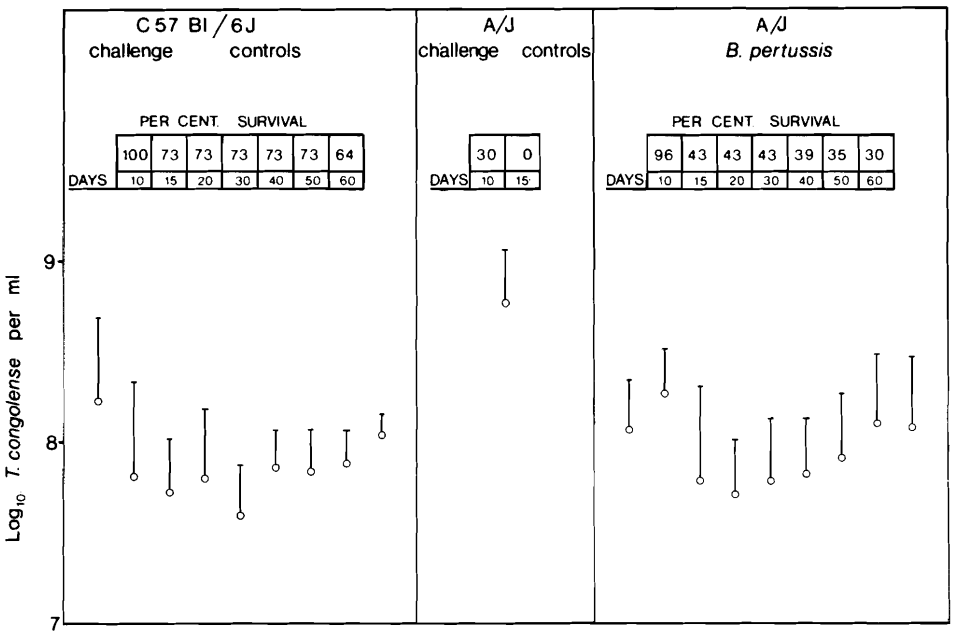


Figure 13. Mean and one standard deviation of 9 peaks of parasitaemia in A/J mice treated with *B. pertussis* and C57Bl/6J mice challenged with *T. congolense*. No difference in the level of parasitaemia was found. Both groups had significantly lower levels of parasitaemia than the A/J-challenged controls.

TABLE 14. X fraction. Vaccine: 2 × 20μg protein B8 at 14-day interval (IP); challenge: 1 × 10^3 B8 or B10, 10 days later (IP)

	Vaccine:Challenge B8 B8	Vaccine:Challenge — B8	Vaccine:Challenge B8 B10	Vaccine:Challenge — B10
Parasitaemia $\log_{10}$ trypanosomes/ml	0	8.2503	8.3793	8.7469
Survival per cent, day 10	100	0	65	20
15	100	0	47	0
20	100	0	40	0
30	100	0	37	0
75	100	0	20	0

C57Bl/6J♀ used

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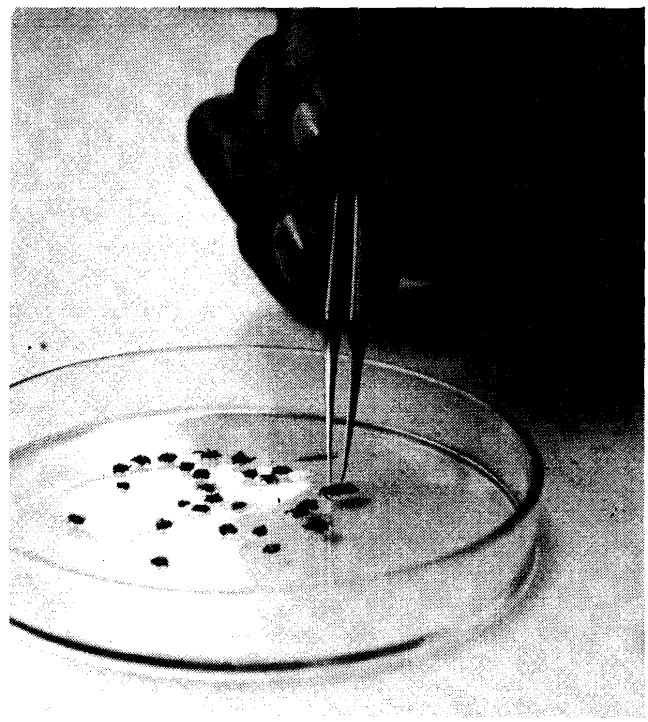
THEILERIOSIS

Theileriosis is a haemoprotozoal infection caused by members of the genus *Theileria* including *T. parva*, *T. lawrencei*, *T. annulata*, and *T. mutans.* The first three appear to be the most important in cattle. In all cases theileria are transmitted by ticks, the distribution of the vector ticks determining the geographic distribution of the disease. *T. parva* and *T. lawrencei* (these may actually be the same species) are important disease pathogens in countries in East, Central and South Central Africa. *T. annulata* causes disease in North Africa, the Middle East, the Indian subcontinent and perhaps elsewhere. *T. mutans* occurs in cattle throughout the world, but is generally not thought to be an important pathogen.

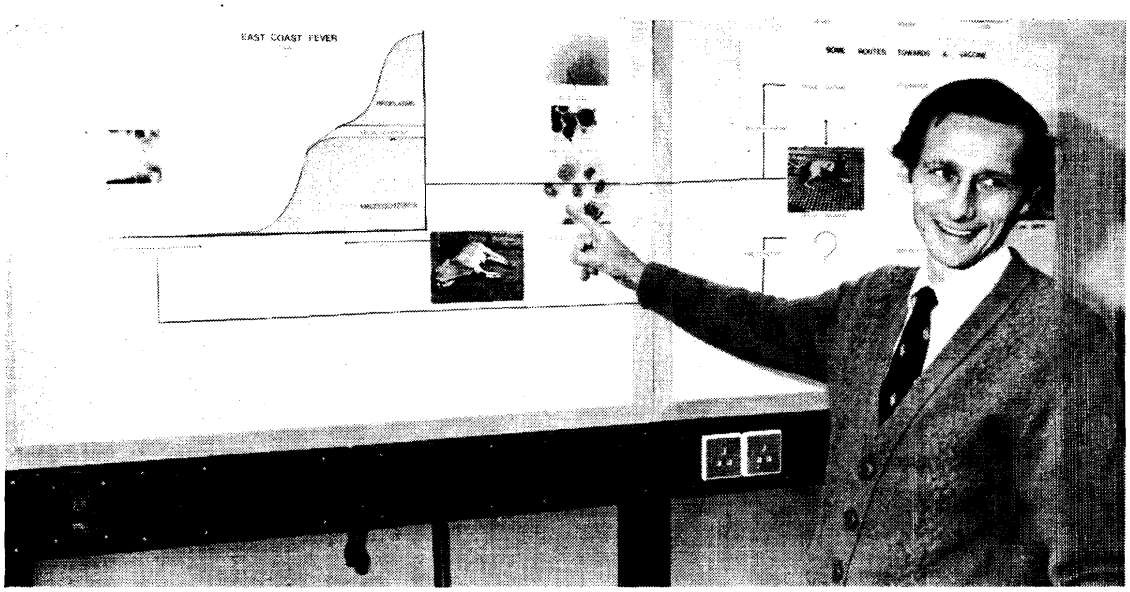
Theileriosis in East Africa frequently causes as high as 80% mortality in susceptible populations of cattle and will cause substantial losses in young stock in endemic areas. Since the malady is transmitted by the bite of infected ticks, intensive efforts must be made to control the vector. To this end animals in endemic areas must be frequently dipped or otherwise treated with acaricide. This is costly in terms of the construction and maintenance of facilities, of acaricide, of staff, and has an important deleterious effect upon the environment. In some areas of East Africa, cattle must be dipped two or three times a week in order to prevent tick infestations and guard against theileriosis. As a result cattle have to trek long distances at frequent intervals with the resultant loss of condition and degradation of pasture.

The ILRAD research activities on theileriosis are oriented toward acquiring the necessary information to ultimately control the disease by immunization. Therefore, the research has a decisive immunologic orientation and involves research on the parasite, the host-defence mechanisms responsible for immunity, the pathogenesis of the disease, serologic and diagnostic procedures and epidemiology. The latter is only now getting under way.



Rhipicephalus appendiculatus **ticks, the major vector of** *Theileria parva*.

An important aspect of the theileria work is cooperative investigations with other institutions. Such cooperative activities have expanded in 1977 and will continue to do so in 1978. That a great deal of expertise and experience in theileriosis (East



Dr. A. Irvin explaining the development of East Coast fever in cattle.

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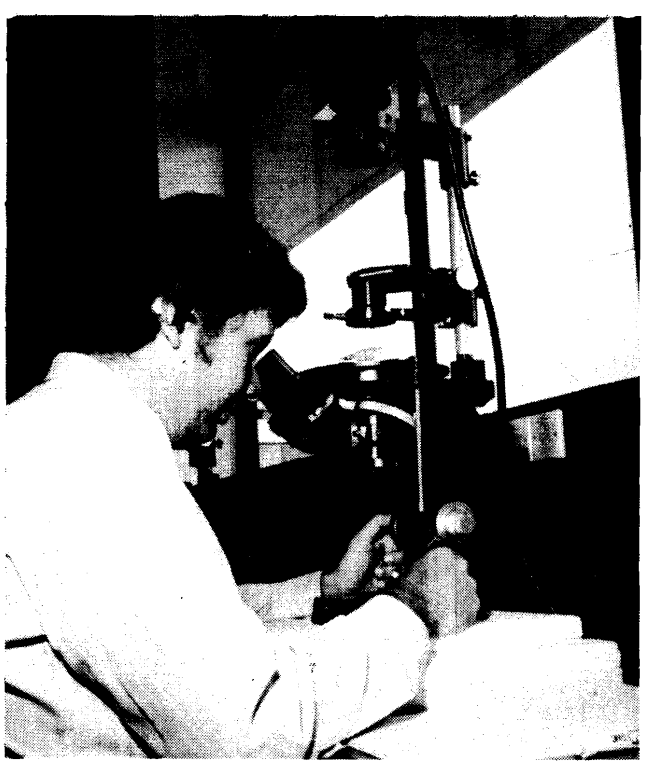
Coast fever) is present in East African institutions is well recognized. ILRAD is fortunate in being located near several of these institutions, such as the Kenya Veterinary Research Department, which makes co-operation easier.

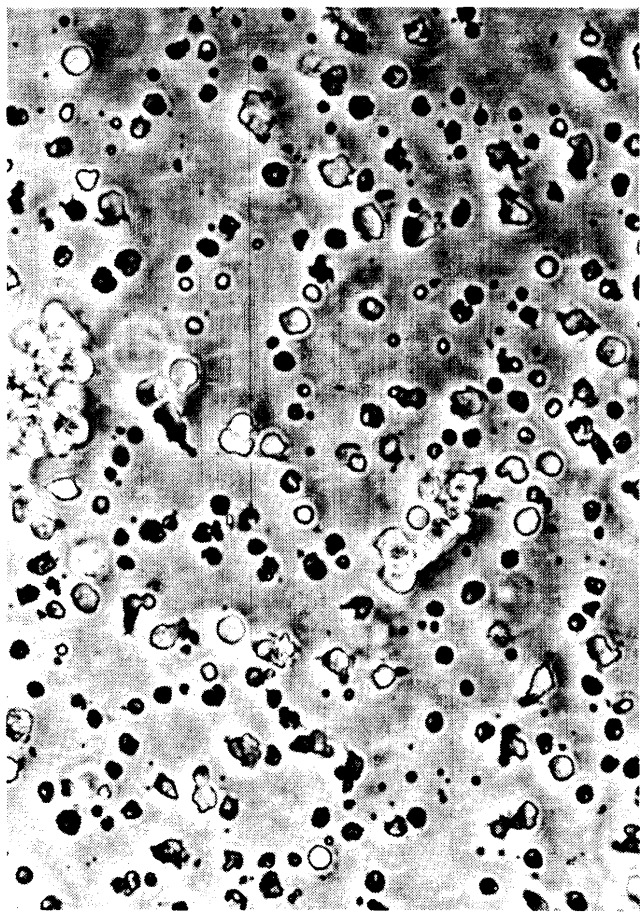
A conference on theileriosis held in December 1976, which was cosponsored by ILRAD and the IDRC, resulted in a publication entitled *Theileriosis*. This publication summarized the proceedings of this meeting and included papers by country representatives, international organizations and others on many aspects of the disease in Africa and elsewhere. This document, along with the reports of various committees, delineates the problem and proposes research approaches including cooperative activities and information dissemination that are relevant to the ultimate control of the disease. It has provided valuable information and has been used in the definition of ILRAD theileriosis research. Such input from countries where the disease is endemic is essential to enable ILRAD research to be oriented towards the 'real' problem as it is experienced by the livestock owner in the field.

PROPAGATION OF *THEILERIA PARVA* IN CELL CULTURES

The overall objective of the theileria cell culture programme is to develop bovine and tick cell culture systems that can be used to propagate the causative organism of East Coast fever, *Theileria parva*. The *in vitro*–grown organisms will be evaluated for their potential as a vaccine against this disease.



Dr. Kurtti examining a culture of *Theileria*-infected lymphoid cells.



Bovine lymphoid cell line persistently infected with *Theileria parva* macroschizonts.

In their life cycle the theileria pass through a variety of cell types within the bovine host and the tick vector, but only certain of the bovine stages, most notably the macroschizonts, can currently be propagated *in vitro*. These macroschizonts can be continuously cultured in lymphoblastoid cell lines and such lines have been demonstrated to have promise for a vaccine. However, the applicability of such material requires considerable immunological scrutiny and for this reason a substantial portion of the cell culture programme is devoted to the procurement and characterization of such cell lines. In addition we are also developing culture systems for the propagation of other stages of the parasite, so that their immunogenic efficacy can be compared with that of the macroschizont-infected lymphoblastoid cells. The cell culture activities have emphasized the following areas of theileria research:

1) The isolation and characterization of lymphoblastoid lines that are infected with different strains of *T. parva* macroschizonts.
2) The evaluation and standardization of culture procedures which are used to procure these lines.
3) The identification of the infective forms of theileria and how they can be manipulated to achieve *in vitro* infection of host cells.
4) The development of culture systems of the tick

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vector, *Rhipicephalus appendiculatus*, that can be used to propagate the tick stages of theileria.

5) The separation of the parasites from the host cell.

Several new lymphoblastoid lines infected with macroschizonts were isolated and their phenotypic traits are being studied, in order to establish delineating characteristics for the lines. The *T. parva* strains that we now have in culture include Muguga, Kiambu and Lawrencei (Serengeti transformed). The original infective material was kindly supplied by the Kenya Veterinary Research Department. The characteristics of the host cells—growth rate, size distribution, karyotypes, and the parasites—morphology, size, number of nuclei, are being catalogued to determine their stability with prolonged subcultivation.

The lines were isolated from the peripheral blood of infected cattle. The leukocytes were separated using flash lysis (hypotonic shock) of the erythrocytes. This method yielded large numbers of viable leukocytes and was suitable for obtaining lymphocytes from the blood of infected cattle for cell cultures. The survival of the lymphocytes *in vitro* was augmented by the use of fibroblast-like cells isolated from peripheral blood, lymph nodes or spleen as feeder layers. As the infected lymphoblastoid cells began to proliferate there was often cytolysis of the feeder layer cells. The nature and specificity of such cytolytic reactions are currently being studied.

The value of using other stages of the parasite in immunization approaches, particularly the salivary gland stage (sporozoite in the tick), needs to be explored. In order to propagate the tick stages we have embarked on a tick cell culture programme. A number of primary cultures from embryos of *R. appendiculatus* were successfully initiated and several new tick lines were isolated. Some of these lines will be selected, characterized and tested for their ability to support the growth and development of *T. parva*. The tick line TTC–243 is also being maintained in our laboratory. However, it has an erratic growth pattern and we are currently examining several environmental and nutritional factors for their influence on the growth of these cells. The susceptibility of the primary cultures and the cell lines to various bovine and tick stages of the theileria is currently being investigated.

T. Kurtti
G. Buscher
M. Pinder
H. Hirumi

PURIFICATION OF MACROSCHIZONTS AND PIROPLASMS OF *THEILERIA PARVA*

Theileria has a predominantly intracellular life cycle and studies on the biochemistry, immunology and infectivity of this parasite are restricted because of the presence of host cell components. It is obvious, therefore, that methods to produce host cell-free parasites are required. Techniques to prepare macroschizonts and piroplasms have been examined as host cells containing these stages can be obtained in large quantities either by *in vitro* propagation (macroschizonts) or following *in vivo* infection (piroplasms).

Macroschizonts: Several methods—ultrasonication, N_2 bombing, hypotonic shock, chemical treatment (SDS) and french press—were examined. Lysis of host cell with a french press gave the best yield of free macroschizonts; however, these lysates were still contaminated with intact cells, nuclei and subcellular debris. Methods for further purification have, therefore, been examined. Much of the subcellular debris can be removed by low-speed centrifugation with minimal loss of macroschizonts. Isopycnic centrifugation did not separate the majority of macroschizonts from the remaining intact cells although this method did produce a nuclear fraction that was 99% pure. However, velocity sedimentation at 1 g separated macroschizonts and host cells, yielding free schizonts with less than 0.01% intact host cells. Some intact nuclei and cytoplasmic vesicles were still present in the schizont fraction. Further improvements to increase the purity of free macroschizonts for biochemical and immunological studies are in progress.

The ability of free macroschizonts purified by velocity sedimentation to infect bovine lymph node cells *in vitro* was examined. To date no evidence of cellular infection was seen although cell-free schizonts remained intact for the first 6 days of culture.

Piroplasms: Peripheral blood of *T. parva*-infected cattle may contain up to 40% piroplasm-infected erythrocytes. The isolation and purification of the piroplasms have been carried out.

Centrifugation of blood on Ficoll-Hypaque gradients removes more than 99% of the leucocytes. Two methods to isolate piroplasms from the purified erythrocytes have been examined. Exposure to hypotonic shock lysed the erythrocytes but the yield of free piroplasms was low. Treatment of piroplasm-infected erythrocytes with rabbit antibovine erythrocyte serum and guinea-pig complement lysed 100% of the host cells and yielded many microscopically normal free piroplasms. Studies on the infectivity and antigenicity of the isolated piroplasms in various cells culture systems and mice are presently under way.

M. Pinder
H. Hirumi

THEILERIA PARVA ANTIGENS

It has been recognized for many years that animals recovered from theileriosis are resistant to reinfection by parasites from the same geographic area. This was shown a number of years ago in early work carried out in South Africa and has since been expanded by research and observations. Investigations carried out at the former East African Veterinary Research Organization indicated, however, that there were antigenic differences between isolates based upon cross-protection in cattle. Thus, the antigenic

specificity of theileria isolates may vary from geographic area to geographic area, but the extent of this variability is not yet known. In addition, the only mechanism presently available to differentiate the antigenic make-up of different isolates is by cattle cross-protection, which is a very expensive and time-consuming activity. As a result, it is necessary to be able to demonstrate protective antigens in theileriosis, to be able to indicate the geographic location of different antigenic types, and to be able to differentiate them by techniques other than cattle cross-protection. Research at ILRAD in theileriosis is being carried out to develop techniques for demonstrating theileria antigens, to develop serologic and other techniques that can be used to show isolate specific antigens, and later to apply such techniques to field and laboratory investigations on the epidemiology and other aspects of the disease.

Fairly early in the clinical course of theileriosis, lymphocytes infected by macroschizonts occur in the draining lymph nodes and in the circulating lymphocytes. Later, destruction of the lymphocytes occurs so that there appears to be a paucity of these cells in the lymphoid system. The mechanism(s) by which infected cells are eliminated from animals that survive the disease is unknown. In other disease systems, it is known that infectious agent-bearing cells frequently have on their surface parasite or agent specific antigens. These can be demonstrated by a number of techniques including immunofluorescence and cell-mediated killing. The research currently under way at ILRAD is using these approaches to demonstrate the presence of cell surface antigens. The results to date, which are incomplete, indicate that there may be specific parasite-associated antigens on the surface of infected lymphocytes. The procedures are based upon raising antibody directed against these antigens and utilizing labelling techniques to demonstrate parasite-associated molecules on the lymphocyte membrane. In addition, attempts are under way to utilize cell recognition phenomena to demonstrate surface alterations and antigens. These studies have not progressed sufficiently to be evaluated. Other studies going on at ILRAD involve the isolation and characterization of the cell surface membranes per se, as well as efforts to isolate macroschizont and piroplasm antigens. These antigens are being used in studies to determine whether strain or isolate specific antigens can be demonstrated and to devise appropriate techniques for laboratory and field application.

A. J. Musoke
L. Rovis
T. W. Pearson
T. Kurtti
T. C. McGuire

SELECTION OF HYPERSUSCEPTIBLE *RHIPICEPHALUS APPENDICULATUS* TICKS FOR *THEILERIA PARVA* INFECTION

In order to have available infectious particles for experimental transmission of *T. parva*, for studies of the immunology and pathogenesis of the disease, it is necessary to obtain infectious particles from *R. appendiculatus* ticks that have been infected by feeding on infected animals. Early in the history of theileriosis research an effort was made to standardize infection by utilizing a 'ten-tick challenge test'. Later Cunningham and coworkers* demonstrated that the grinding of infected ticks could result in a stabilate, which could be preserved and standardized after titration in susceptible cattle, for many aspects of theileriosis work. Recent investigations here in Kenya have shown that the infection rate of *T. parva* in *R. appendiculatus* reached only 30% even when the ticks had been fed on cattle with high parasitaemias. These results, as well as the observations of others, have also shown that there is a great divergence in the number of salivary gland acini infected among different ticks fed upon the same animal. In order to be able to provide ticks that have a high infection rate from which a large number of infectious particles can routinely be obtained, it would be of benefit to have at hand a strain of ticks that could be routinely infected to a high degree. The work described in this report relates to efforts made to select ticks that are highly susceptible to infection with *T. parva*.

The investigations were based upon the infection of nymphs on infected calves, followed by examination of the adult ticks three to five days after moulting to determine the presence of infection by the examination of haemolymph and gut for zygotes and kinetes of *T. parva*. Positive ticks were used for further breeding and the negative ones were eliminated. After the positive ticks had fed on rabbits and the engorged female ticks had left the host, they were placed in an incubator for oviposition and hatching of larvae.

The larvae were again fed on rabbits and moulted to nymphs after leaving the host. The nymphs represented the second generation and were again fed on infected calves and the adults examined for infection by the demonstration of zygotes and kinetes of *T. parva*. This procedure was repeated for three generations of ticks. At the same time the salivary glands of the male ticks were examined for infection with the developmental stages of *T. parva* by staining of impression smears. After the third generation a quantitative analysis of the infection rate in the acini of the salivary glands was conducted, using the Feulgen staining technique following dissection of the whole salivary glands from the tick.

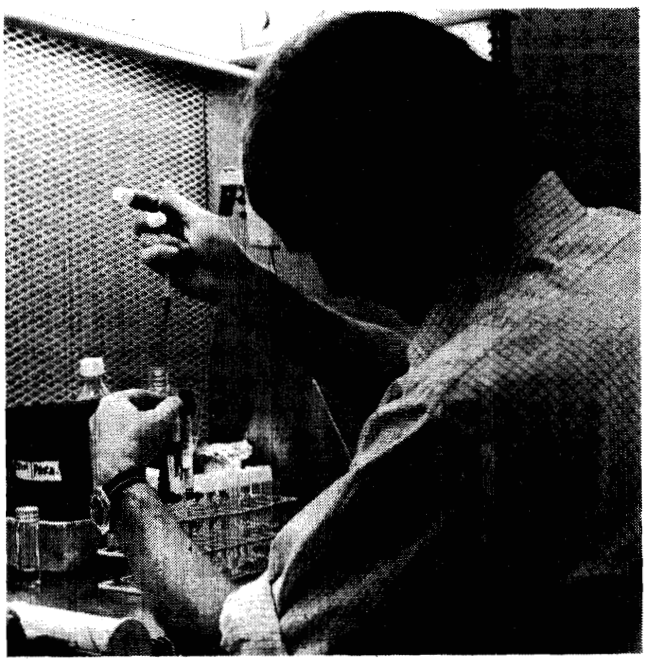
The results of the trials indicated that it was possible to select ticks with an increasing level of infection as measured by the number of kinetes and zygotes in the haemolymph. When the females were

*Cunningham, M .P., Brown, C.G.D., Burridge, M.J., Musoke, A.J., Purnell, R.E., Radley, D.E., and Sempbwa, C. 1974. East Coast fever: titration in cattle of suspensions of *Theileria parva* derived from ticks. *Br. vet. J.* 130:336–343.

further examined, however, it was found that there were a large number of acinar cells infected and that the females reproduced very poorly. This was thought to be due to the high degree of infection in the salivary glands which caused cell necrosis and probably interfered markedly with feeding. Therefore, a decision was made to select only infected males rather than females. The results further indicate that it is possible to select ticks with a very high rate of salivary gland infection and that the infection can become sufficiently severe to be detrimental to the ticks themselves.

The research is continuing along the lines of selecting infected male ticks that will be bred to non-infected females in order to establish a high percentage of infected ticks, each of which contains a large number of infected acinar cells per salivary gland. The results obtained to date further indicate that it is possible to select hypersusceptible ticks and that such a selection process should make it possible to provide infected ticks with a large number of sporozoites for stabilate production and for use in other research on theileriosis.

This report covers cooperative research activities between ILRAD and Dr. E. Schein and Dr. M. Warnecke of the Institute of Parasitology and Tropical Veterinary Medicine of the Frei University of Berlin.



Research assistant Clifford Boarer separating bovine lymphocytes for transformation studies in East Coast fever.

PATHOGENESIS

The pathogenesis of theileriosis, at present poorly understood, is an important component of the total information upon which ultimate control of the disease can be based. Such information, amalgamated with a knowledge of the immunology of the disease including the effector mechanisms for host resistance, will be the major criteria dictating approaches to be carried out for protection against challenge in the field.

The chain of events potentially leading to the death of animals from theileriosis begins by introduction of infectious particles into a susceptible host by the bite of an infected tick. These particles will have previously developed in the salivary glands of *Rhipicephalus appendiculatus* ticks and are introduced into the host at the time of tick feeding. After introduction, the organism undergoes unknown developmental sequences and can initially be observed as macroschizonts in the lymphocytes of lymph nodes draining the site of the bite. Later, the number of lymphocytes infected with macroschizonts increases in number and macroshizonts can be detected in other lymphoid tissues and in circulating lymphocytes. Later, the parasites can be observed as piroplasms in the erythrocytes. Ticks can subsequently be infected by feeding and ingesting a blood meal containing erythrocytes with piroplasms. Animals generally succumb to the disease approximately three to four weeks after initial introduction of the organism.

Research carried out by others suggests that the protection of the animal against challenge can be provided by parasite antigens during premacroschizont developmental stages, as well as during the macroschizont stages. This finding is based upon immunization procedures carried out for *T. parva* at the former East African Veterinary Research Organization in Muguga, Kenya. The immunogens that are responsible for host protection during any of these stages of parasite development are unknown.

The ILRAD research activities on the pathogenesis of theileriosis are being carried out on animals experimentally infected with *T. parva*. In addition to the tissue and fluid alterations in the diseased animals when clinically ill, the early stages of the infection including the tick bite site are being examined. Included are efforts to appropriately tag the infectious particle so that it can be followed within the tissues of an experimentally infected animal. Both fluorescence microscopy and radioisotopic labelling techniques are being used in these studies. The investigations have not yet proceeded sufficiently to provide a complete picture of the pathogenesis. This research is being currently emphasized and will be expanded by additional ILRAD staff that will be recruited in the near future. It is anticipated that such studies will define the mechanisms by which the disease is produced and the location, in the chain of events leading to disease and death, where protection can be most effectively induced.

J. B. Henson

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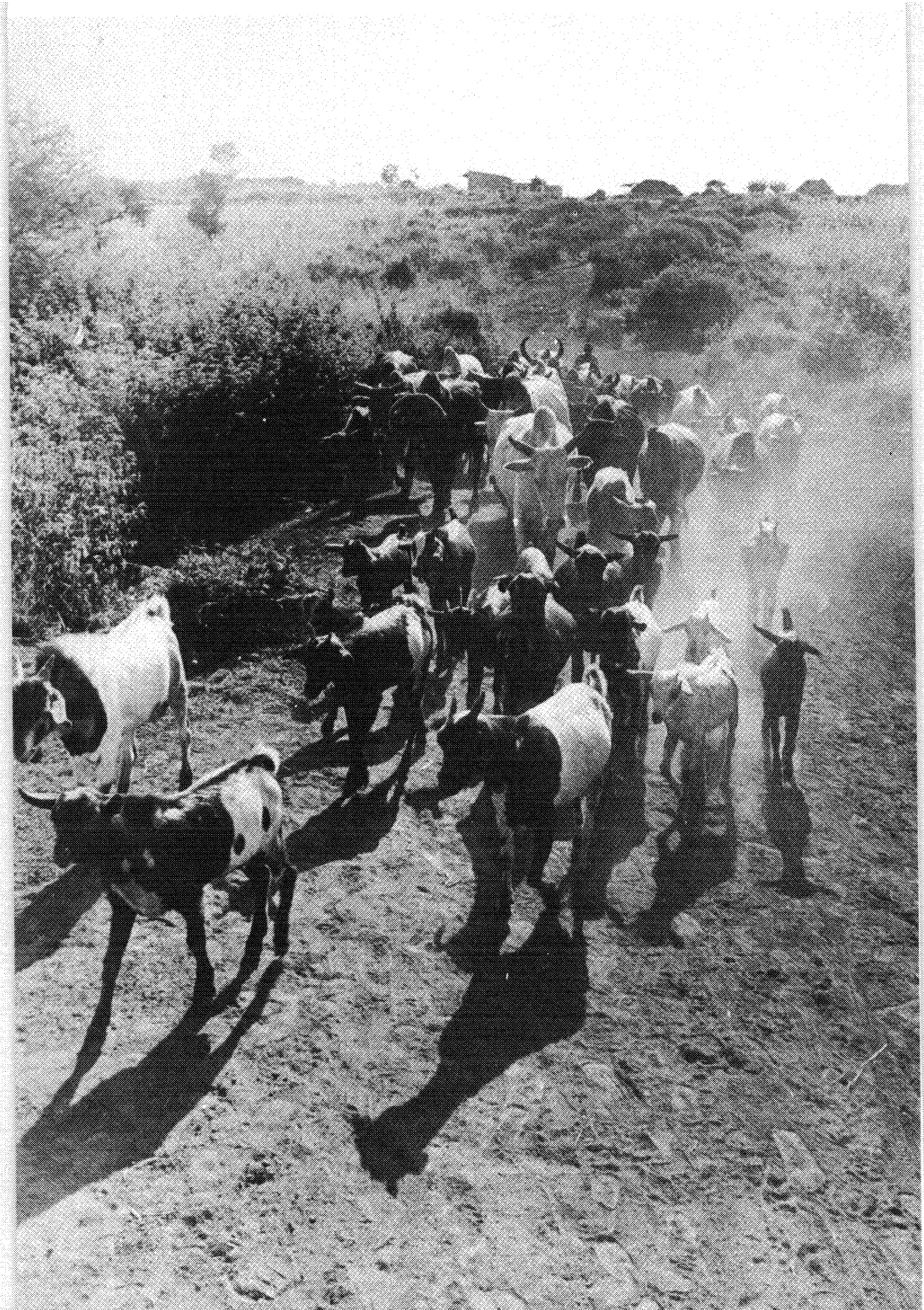


BOVINE IMMUNOLOGY

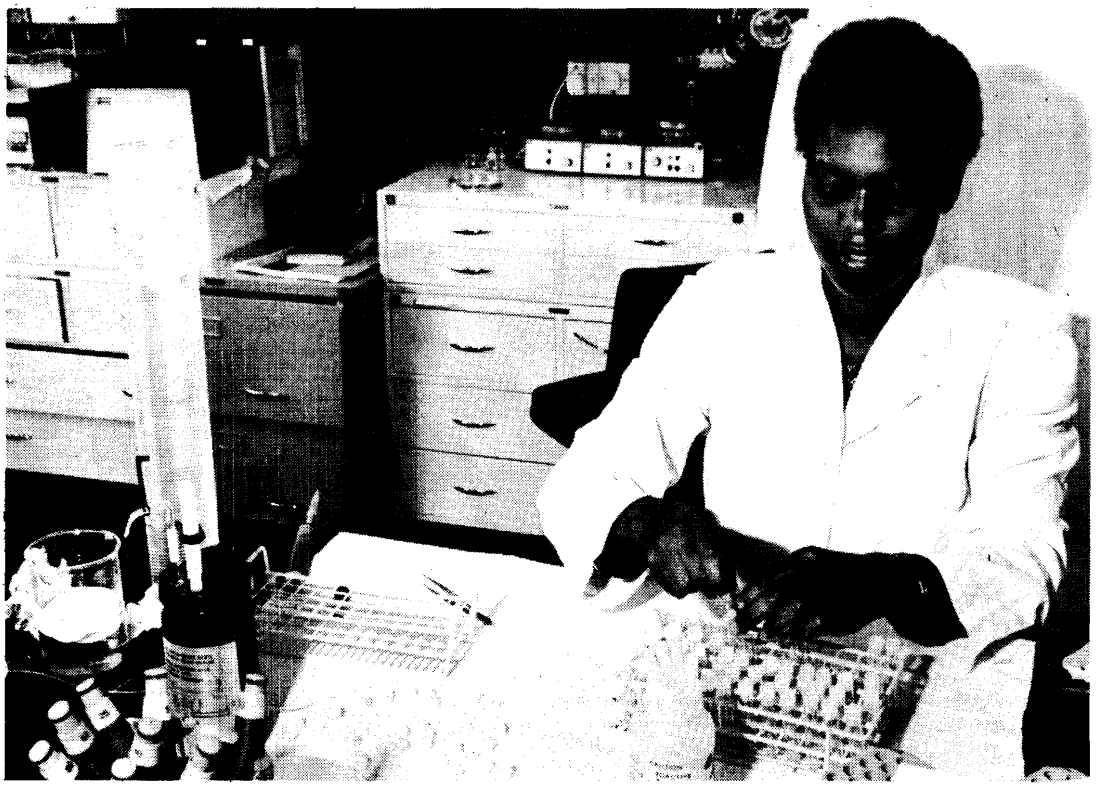
The immunology of ruminant animals and an understanding of the functions and composition of their host defence processes are of paramount importance in combating trypanosomiasis and theileriosis. It is recognized that there are differences in the immune systems of various species of animals, although there are many similarities. Information concerning the immunology of ruminants is very scanty in contrast to the voluminous information available on mice and man. As a result of this deficiency and as a requirement for techniques applicable to the bovine immune system, it is imperative that ILRAD researchers as well as other scientists develop a good understanding of the immune system in the bovine and other ruminants and define the optimal effector mechanisms for destruction of trypanosomes and theileria.

Research at ILRAD is directed to both antibody and cell functions in the bovine. It is necessary to develop a basic understanding of bovine immunoglobulins, immunoglobulin classes and subclasses and their biological functions. The research will then be directed to determine which of these classes or subclasses is most effective in killing the organisms under investigation.

Likewise, cellular immunology is poorly understood in the bovine. It has been necessary to develop technology to be able to define the normal cell populations in the lymphoid system of cattle, and then direct the research activities to relating the cell interactions in the immune response and how these function in theileriosis and trypanosomiasis. This research is currently under way and is presented in the following report.

FUNCTIONAL ACTIVITIES OF BOVINE ANTIBODIES

Initial studies with bovine immunoglobulins were to define their biologic properties, especially those of the known IgG subclasses, IgG_1 and IgG_2. To accomplish this, antibodies to defined antigens were made, separated by affinity chromatography, and the various classes and subclasses purified. Cattle antibodies to horse erythrocytes were reacted with horse erythrocytes and subsequently eluted by increasing the temperature to 56°C. Eluted IgM antibodies were separated by gel filtration, while IgG_2 antibodies were obtained by ion exchange chromatography. IgG_1 from ion exchange columns was always contaminated with IgG_2 which could be removed on isoelectric focusing columns. Cattle antibodies to ovalbumin were separated from serum on ovalbumin-agarose columns and eluted with an acidic buffer. The cattle antibodies to DNP were isolated on DNP-lysine-agarose columns and eluted with excess DNP. IgG_1 and IgG_2 fractions to each antigen were separated by the same techniques used for antierythrocyte antibodies.



Jane Ngaira performing a radioimmunoassay.

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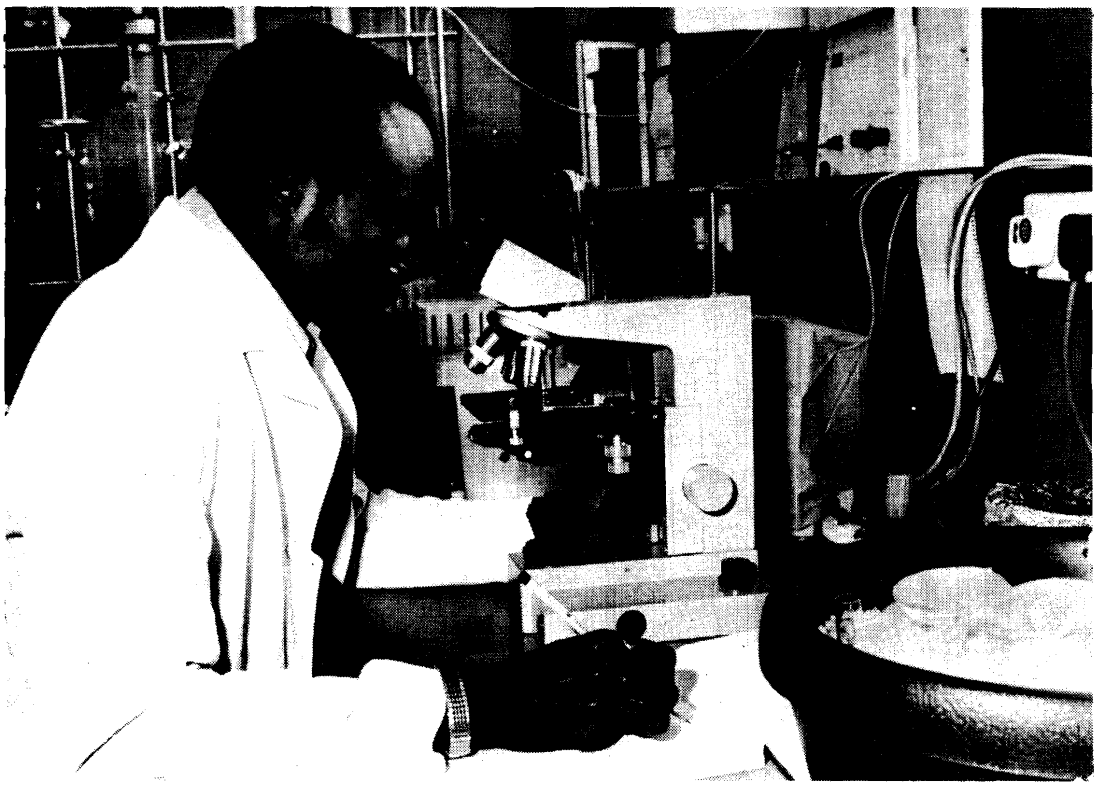

Dr. A. Musoke estimating the number of viable lymphocytes for surface immunoglobulin staining.

Isolated bovine peripheral blood monocytes were reacted with bovine antibody-coated horse erythrocytes and observed for adherence and phagocytosis. Approximately 90% of the monocytes phagocytized IgG_2-coated erythrocyte, demonstrating a receptor for IgG_2 antibody. Monocytes did not react with IgM-coated erythrocytes, except after the addition of complement showing the absence of an IgM receptor and the presence of a complement receptor. It should be noted that bovine complement would not work in this system and that equine complement was used. Perhaps conglutinin in the bovine complement source blocks the reaction. IgG_1-coated erythrocytes failed to mediate significant adherence and phagocytosis, either with or without complement. These data suggest the absence of an IgG_1 receptor on peripheral blood monocytes. When lung macrophaques were tested, both IgG_1- and IgG_2-coated erythrocytes were bound and phagocytized. The difference between receptors on the various phagocytic cell types for cattle IgG subclasses is being further characterized. Especially important is the interaction between phagocytic cells and IgG_1 and IgG_2 antibody-coated trypanosomes. Antibodies purified as described above were also tested for their ability to fix bovine and guinea-pig complement. Antigen (ovalbumin or DNP-carrier) and antibody (IgG_1 or IgG_2) complexes were formed following described methods. The indicator system for the bovine complement consisted of rabbit cells sensitized with sheep haemolysin, while for the guinea-pig complement, sheep cells sensitized with rabbit haemolysin were used. Both IgG_1 and IgG_2 complexes of either ovalbumin or DNP carrier were capable of fixing bovine complement, but IgG_1 complexes were consistently more efficient than IgG_2 complexes by at least 20%. On the other hand, when guinea-pig serum was used as source of complement, fixation was demonstrable only with IgG_1 complexes of either antigen. Complexes of IgG_2 did not fix guinea-pig complement in any appreciable amounts. This latter observation supports results obtained by other workers.

Efforts to identify short-term fixing antibodies in the IgG class are under way. Preliminary data indicate that this activity is associated with both IgG_1 and IgG_2 subclasses.

Since only the variant specific surface antigen (VSSA) appears to be exposed on live trypanosomes we plan to study antibodies to this antigen. Cattle were infected with a clone of *T. brucei* and sequential sera taken. The purified VSSA of this clone is being attached to agarose. Specific antibodies will be obtained, separated into classes and subclasses, and their interaction with live trypanosomes studied. Included will be the interaction of phagocytic cells and complement.

T. C. McGuire
A. J. Musoke

BOVINE LYMPHOCYTE MEMBRANE ANTIGENS: CHARACTERIZATION AND ANALYSIS BY MONOCLONAL ANTIBODIES

In order to understand many aspects of the immune response it is often necessary to understand the types and functions of its component parts. In mammals, lymphocytes are the prime effector cells in most

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immune responses, and comprise a functionally heterogeneous mixture. Unfortunately, lymphocytes with different functions are often similar in morphology and various other physical parameters and thus it is difficult to isolate (or even identify) lymphocyte subpopulations. One approach to this problem is to use antibodies that recognize specific differentiation antigens and thus discriminate between lymphocyte subpopulations. This technique obviously depends on the availability of antisera that recognize only the desired antigenic determinants and has been most effectively used in the mouse, where inbred strains are available and allow the controlled production of antisera with appropriate specificity.

Genetically defined bovids are not available, thus dissection of bovine lymphocyte populations using antisera is extremely difficult. However, a new technique, developed by Köhler and Milstein* in Cambridge, England, allows a radically new approach for the manufacture of specific antibodies. In essence, tumour cells are fused with lymphocytes from an animal that has been immunized with a specific antigen (for example, bovine lymphocytes). The resulting hybrid cells acquire properties of both parental cells, that is, the ability of the tumour cell to grow indefinitely *in vitro* and the ability of the immune lymphocyte to produce antibody specific for the antigen used to immunize the donor animal. The hybrid antibody-producing cell is then cloned and the resulting hybrid cell by definition secretes a 'monoclonal' antibody. In this way, antibodies specific for a single antigenic determinant can be made and the cells that produce them are 'immortalized'. The specificity of the antibodies obtained is thus determined by the selection of appropriate antibody-producing clones. The immunizing antigen need not be pure.

We have recently used the monoclonal antibody technique to produce antibodies to bovine lymphocyte surface antigens. We produced 42 different hybrids which secreted antibody directed against bovine lymphocyte surfaces. Of these 42 hybrids 36 produced antibody that can be detected in a binding assay but showed little cytotoxic activity, 5 produced antibody that was cytotoxic and gave little or no binding, and one hybrid produced antibody that was strongly positive in both the binding and cytotoxic assay. Antibodies that were strongly positive in the binding assay (12) were examined by immunofluorescence microscopy; 5 of these were directed against antigens present on the majority of bovine peripheral blood lymphocytes, whilst the remainder detected antigens present only on subpopulations. At the present time, 8 of these hybridomas have been successfully cloned and secrete specific antibody. Analysis of antigens detected by these antibodies is under way, and it is anticipated that these antibodies and ones produced subsequently will define functional bovine lymphocyte subpopulations in normal and infected animals.

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*Köhler, G., and Milstein, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256:495.

BINDING AND STIMULATION OF BOVINE PERIPHERAL BLOOD LYMPHOCYTES BY LECTINS

Before initiating a programme to study immune reactions in cattle infected with trypanosomes or theileria it is necessary to establish a 'baseline' for various parameters of bovine lymphocyte reactivity.

As a start we have studied the binding and stimulation of bovine peripheral blood lymphocytes by various lectins. Of the 11 lectins tested, 6 induced stimulation of DNA synthesis. Concanavalin A, succinyl-Concanavalin A, phytohaemagglutinin M, pokeweed mitogen and bacterial lipopolysaccharide were mitogenic under conditions similar to those found optimal for murine lymphocyte stimulation. Peanut agglutinin (PNA) stimulated bovine lymphocytes whereas it does not stimulate normal mouse, rat, guinea-pig or human lymphocytes. Soya bean agglutinin, wheat germ agglutinin, succinyl-wheat germ agglutinin, *Ulex europeus* agglutinin and *Helix pomatia* agglutinin were not mitogenic.

The binding of 5 lectins was examined by fluorescence microscopy together with the binding of an antibovine Ig reagent. Peanut and soya bean agglutinin bound to a large proportion of Ig negative lymphocytes. Wheat germ agglutinin bound to a proportion of Ig^+ as well as Ig^- lymphocytes. Concanavalin A bound to most lymphocytes, while *Ulex europeus* bound to none.

It is interesting that lectin binding might define bovine lymphocyte subpopulations. PNA might be a particularly interesting marker for bovine T cells. Moreover, changes in stimulation by lectins might be used to monitor suppression of subpopulations of lymphocyte reactivity in disease states.

In limited experiments with *Theileria parva*-infected animals, it appears (*a*) that the macroshizont-infected lymphoblasts are Ig^-, PNA^+ cells, and therefore may have arisen from a common cell type, and (*b*) that the stimulation of peripheral blood lymphocytes is greatly diminished, possibly demonstrating immune suppression similar to that observed in trypanosome-infected mice.

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CHARACTERIZATION OF THE THY.I, TL AND IG PHENOTYPES OF MOUSE PEANUT AGGLUTININ POSITIVE LYMPHOID CELLS

The Thy.1, TL and Ig phenotype of PNA-binding cells was characterized in various strains of mice. In the thymus, PNA was found to bind principally, but not exclusively, to the Thy.1$^+$TL$^+$Ig$^-$ steroid-sensitive cortical thymocytes. Thy.1$^+$TL$^-$Ig$^-$ steroid-resistant cells are not labelled with PNA. In other lymphoid organs, PNA bound to a minority of T or null cells, not to B cells.

During ontogeny PNA$^+$ and PNA$^-$ T lineage cells appear simultaneously in the liver at day 10 of gestation, in the thymus at day 13 and in the spleen at day 18. No evidence was found for a maturation from PNA$^+$ to PNA$^-$ cells. Prethymocytes present in nude mice were also divided into PNA$^+$ and PNA$^-$ populations.

The findings support the proposition that cortical and medullary thymocytes develop independently. Moreover, they suggest that both populations might possibly arise from distinct precursors and give rise to distinct functional peripheral T cells.

In view of the fact that PNA binds to Ig$^-$ lymphocytes in the bovine, it is believed that the characterization of the phenotype of mouse PNA-binding cells may shed some light on the nature of these cells in the bovine, where alloantigen markers are not available as yet.

G. E. Roelants
K. S. Mayor-Withey
J. London

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RESEARCH SUPPORT

A number of research support facilities are provided at ILRAD. They include Clinical Medicine and Diagnostic Services, Biological Services, Central Dish Washing and Media Preparation, the Farm, and the Central Laboratory Animal Facility. All of these have important and necessary functions in support of the research activities. Details of the varied research support activities will not be elaborated here, but a limited amount of information will provide some appreciation of what is entailed.

Joseph Odero Oyugi in the central dishwashing facility.

THE CLINICAL MEDICINE AND DIAGNOSTIC SERVICES

Clinical medicine and diagnostic services were established to provide supportive capabilities for the overall research programme at ILRAD. Activities of the section include the acquisition of all experimental animals, operation of a diagnostic laboratory facility and a clinical-surgical unit, as well as research expertise in the areas of nutrition and clinical medicine.

During the past year considerable effort has been made to initiate a calf-rearing programme at the institution to provide the scientific staff with the best possible bovine animals for their research programmes. To date this effort has successfully reared 60 calves and it is anticipated that by 1979 ILRAD will be rearing in excess of 200 calves per year. Coincident with this programme are plans to develop the overall farm operation substantially to include a breeding herd, forage harvesting capability, and significant improvement and expansion of the farm animal holding facilities. The new laboratory animal facility has begun operation and is starting to breed rats, mice and rabbits. it is anticipated that within the next few years all experimental animals will be bred and reared at ILRAD.

The success in finding an effective prophylaxis against trypanosomiasis and theileriosis greatly depends on the availability of experimental cattle that have not been exposed in the field to either of these two diseases. In order to evaluate the disease status of cattle prior to their assignment to a specific experiment, rather extensive serological screening has been necessary and has become a routine exercise in the diagnostic laboratory. To date over 2000 cattle on six different commercial enterprises have been serologically screened for haemoprotozoal infections. Less than 10% of these cattle were found to be serologically negative for all antigens tested. Standard serologic techniques such as agglutination, indirect fluorescent antibody, indirect haemagglutination and ELISA (enzyme-linked immunosolvent assay) are presently being utilized. In addition to the serological screening, bovine, caprine, and ovine animals are periodically checked for the presence of brucella, salmonella and clostridial organisms, and all breeding laboratory animals are routinely screened for over a dozen potential pathogenic organisms including micoplasma. The laboratory also has the capability of assisting in routine diagnostic procedures in conjunction with the pathology department and the clinical medicine staff.

Since both trypanosomiasis and theileriosis have a significant effect on the host's haemopoietic system, and as an aid to the clinical medicine staff, the diagnostic laboratory is also developing a haematology section, which will provide service on a routine basis to all scientific staff who require monitoring of specific haematological parameters during the course of a given experiment.

Obtaining experimental material from living animals for the scientific staff is the responsibility of the clinician in the section, with lymph node removal, splenectomies, caesarean section, etc., presently carried out on a routine basis. The clinical monitoring of all experimental and non-experimental animals at

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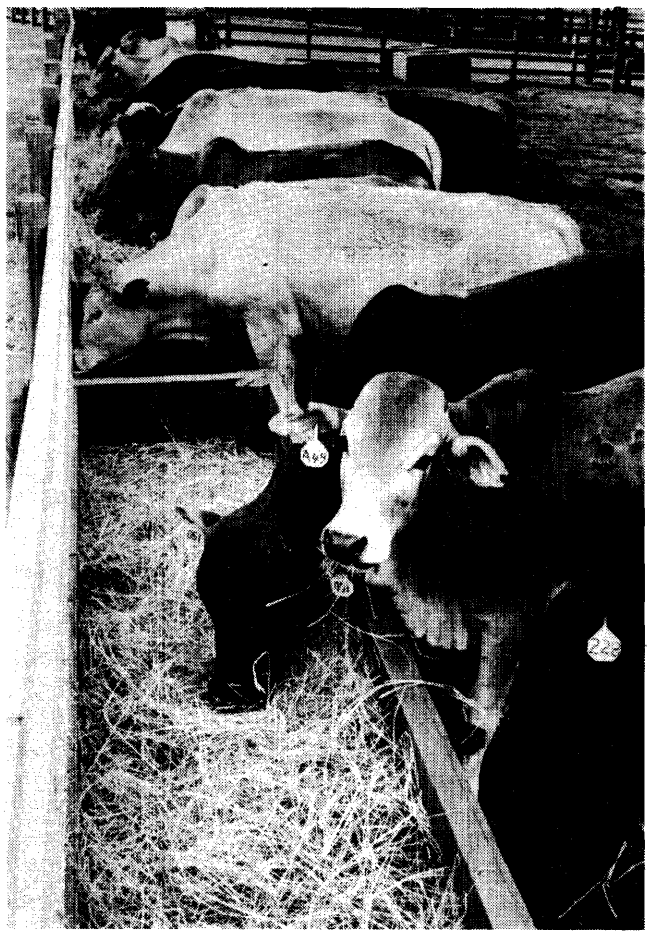
Clinical assistant George Ngekenya and senior animal attendant Ezekiel arap Meli check an animal before an East Coast fever experiment.



Cattle being prepared for the production of *Theileria*-infective material.

ILRAD is done on a 24-hour basis and complete medical records are maintained.

Although supportive service is the major function of the section, collaborative research is presently under way with various staff members. At the present time these activities involve a detailed study of the effect of plane of nutrition on various haematological parameters in bovine trypanosomiasis, and a detailed clinical evaluation of animals infected with East Coast fever.

John Tangus of the tick facility performing a biopsy of the parotid gland to search for schizonts in lymphocytes.

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BIOLOGICAL SERVICES

Biological Services involves the trypanosomiasis and theileriosis stabilate bank, as well as the provision of other biological materials for research activities. Trypanosomes and theileria are subjected to slow freezing to −196°C in the presence of protective agents. The slow freezing of these organisms, in either blood or other materials, allows the viability of the parasites to be retained and allows them to be stored for long periods in the frozen state.

By this technique it is possible to collect organisms sequentially during the course of disease, or to prepare large batches of material to be used for a long time in the research activities. An example would be the preparation of 2000 ml of theileria stabilate which would be stored in 1 to 2 ml samples. After appropriate evaluation, this material would serve as a standardized source of infectious particles for a variety of research activities. Similarly, different primary isolations and stocks can also be stored and maintained in containers of liquid nitrogen.

The scientist indicates to Biological Services the stock and amount of organism required, and for what period, and Biological Services prepares the material. This allows for standardization of the material and also enables the scientist to utilize his time as effectively as possible. In addition to infectious materials, Biological Services provides cells and tissues from animals as required. An example of such a requirement would be the provision of thymus for work on defining cell surface markers on bovine embryo thymus.

CENTRAL LABORATORY ANIMAL FACILITY

The Central Laboratory Animal Facility is the support unit responsible for the provision and maintenance of laboratory animals used in the research programmes. Included are mice, rats and rabbits.

The unit occupies the recently completed facilities that provide animal and support rooms, as well as a centralized cage washing and sterilization capability. Rooms for feed and other storage and experimental manipulation of laboratory animals are also provided. The animal rooms are individually temperature and air exchange controlled, so that a defect in one of these units will not result in the total facility ceasing to operate until the mechanical equipment can be repaired.

Included in the functions of the unit are the breeding of rats and mice to provide these animals for ILRAD researchers. At the present time most of these requirements are being met from other sources as the ILRAD breeding colonies are now being built up to meet the total needs of the institution. The breeding of rabbits, which will be initiated soon, requires some additional facilities that are now being planned. The Central Laboratory Animal Facility is a modern, functional, laboratory animal unit with the necessary support and production capabilities to provide high quality animals for ILRAD research. In addition, the unit has already been, and will in the future be used for the training of laboratory animal staff. The supervisory staff in the unit also participate in laboratory animal management training at other Kenyan institutions.

THE FARM

The ILRAD estate comprises 180 acres of land, most of which is divided into paddocks for animal use. In addition, a number of domestic animal facilities have been constructed on site for maintaining the animals used in research.

Robert Mburu of the central laboratory animal facility checking the progress of mice undergoing experimentation.

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Eleven-week-old Hereford cross calves being reared at ILRAD for future experimental use.

The responsibility of the farm staff is to procure and maintain the domestic species—cattle, sheep and goats—required for the research activities. Paddocks, open pens, dips and crushes are provided to manage large numbers of cattle, sheep and goats. Experimentally infected animals are maintained in isolation buildings under screened conditions to preclude access of flies and other insects. The rearing of newborn calves to weaning age and the maintenance of these animals in isolation to prevent exposure to other diseases are a part of the farm activities. It is anticipated that the number of new-borns reared will increase in 1978 and 1979. These calves, raised in isolation, are then used in selected experiments. Also included in the farm activities is the acquisition either on site or from other sites of the necessary forage and other feed stuffs for the domestic animals.

The ILRAD farm continues to develop to meet the needs of the institution and offers a spectrum of activities relating to the varied research animal needs of the institution.

The ILRAD complex at Kabete.

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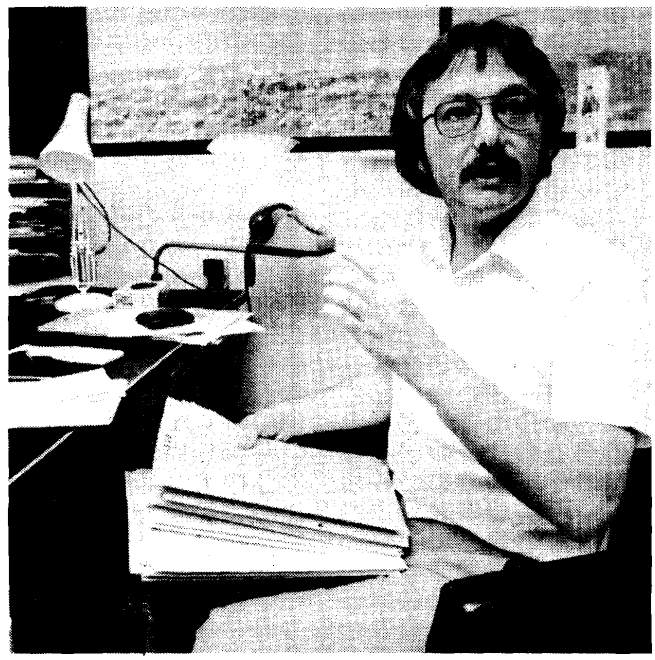
CONFERENCE, TRAINING AND INFORMATION IN 1977

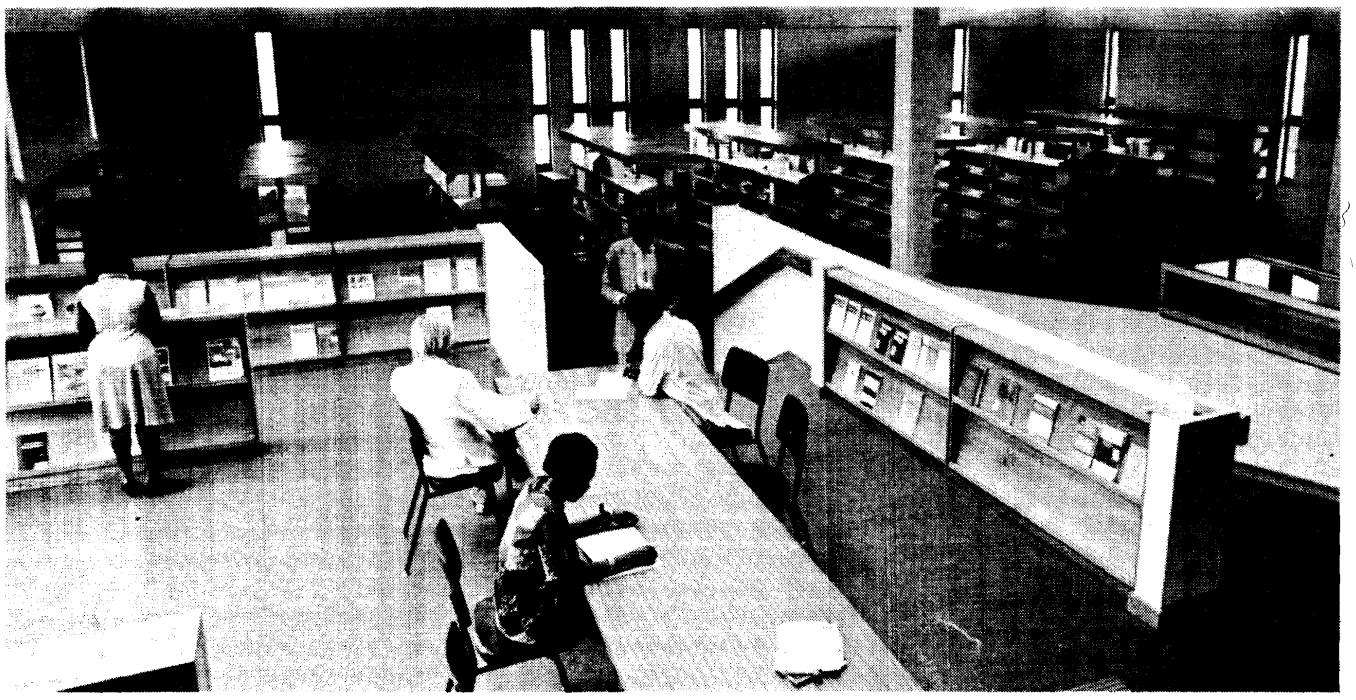
If the research undertaken at ILRAD is to make a meaningful contribution to the sum of knowledge on and control of theileriosis and trypanosomiasis, it is important that the institution be in the closest possible contact with others working in the same field, or on associated problems. Accordingly, a very important function of ILRAD is to act as a centre for information diffusion, not only to other scientists and research establishments but also to national and international decision-makers, in both the developed and the developing world. The shortage of highly trained personnel in the developing world necessitates an appropriate response from ILRAD to ensure that the assistance offered is both relevant and useful to those working at all levels, often under extremely difficult conditions.

There are three maior ways in which ILRAD undertakes the task of information diffusion: the acquisition of relevant data accruing from the ILRAD research programmes, the training of personnel, and the general dissemination of information relating to trypanosomiasis and theileriosis. ILRAD research results will be made available to the general scientific community via publication in scientific journals, symposia and proceedings. The organization of and participation in national and international conferences and seminars brings the work of the institute to a wider audience, and also provides valuable information for ILRAD scientists. The participation of representa-



Dr. Max Murray, chairman of the ILRAD training committee, explaining the training programme.

The ILRAD library.



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tives from those parts of the developing world directly experiencing the problems provides a valuable insight into the day-to-day reality of disease control and helps to define the problem in ways that are relevant, both to ILRAD and to other national and international institutions. Close cooperation at all levels prevents the waste of resources on duplication of effort. In 1977 a large number of scientists from developing and developed countries visited ILRAD. In 1977 representatives of ILRAD participated in over 20 different conferences. Of specific interest was the 15th Meeting of the International Scientific Council for Trypanosomiasis Research and Control, held in The Gambia.

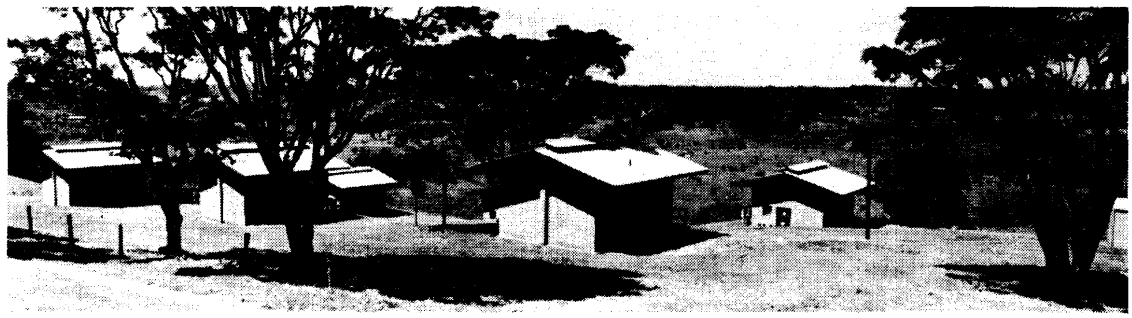
Training in all its aspects is considered an integral part of the function of ILRAD. Its international staff represent an important concentration of expertise in Africa in the fields of parasitology, immunology, pathology, biochemistry, cell culture and other disciplines. Not only can trainees acquire newly developed techniques, but training can also be provided in the basic techniques relating to many aspects of animal disease research. The ILRAD training programme is designed to counter the shortages of trained personnel in the developing world, and to produce individuals capable of training others, so as to strengthen the national institutions and form a basis upon which the work of ILRAD can be applied to the problems in the field.

ILRAD accepts postgraduate students in disciplines relevant to its research programme and mandate. Courses and individual instruction are designed to complement the teaching of the student's home institution with considerable emphasis placed on practical as well as academic aspects. Graduates from developing countries are eligible for a number of ILRAD fellowships, whereas those from the developed world are expected to be financed by their home institutions. A number of postdoctoral students also worked at ILRAD in 1977, and while designated as scientific staff also received in-service training. Individual instruction was given to trainees and scientists from developing country institutions in relevant activities.

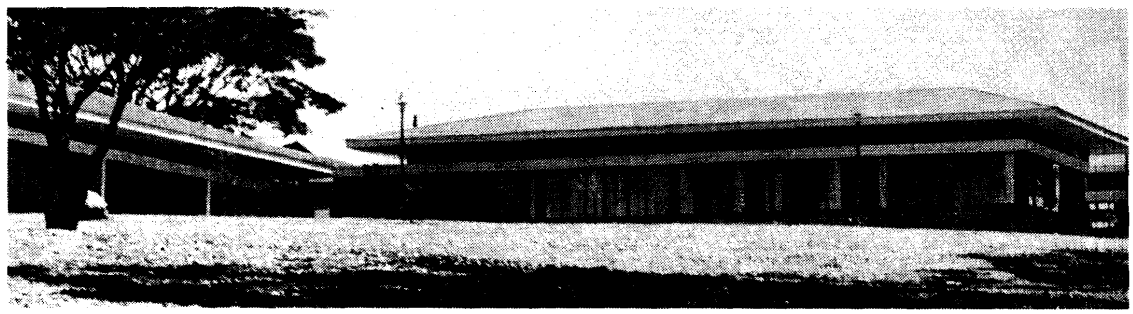
ILRAD is concerned with the organization of a range of training courses and seminars in both the applied and basic sciences. In September 1977, a Leadership Training Course on African Trypanosomiasis was held in Nairobi under the joint sponsorship of the OAU, ILRAD, FAO and the WHO. The 71 participants represented 31 African states, and the training reflected a strong desire to relate the problem of trypanosomiasis to the local environment and to laboratory and field skills necessary for trypanosomiasis research and control. Other training courses, in the process of organization for 1978, include a 6-week course on the use of radioactive isotopes, cosponsored with IAEA, and a course on tissue culture techniques for parasites, in association with the WHO and the FAO. In addition, ILRAD staff hold regular open seminars locally and lecture in colleges and universities in Kenya. At the technical level ILRAD is examining the possibility of providing training to a standard equivalent to the Fellowship of the Institute of Medical Laboratory Technologists. Great emphasis will be placed on the fact that such trainees should eventually be capable of training others in their home institutions, and accordingly ILRAD will adopt a flexible approach as the best response to the specific needs of the trainees.

Both the ILRAD research activities and the training programme require a comprehensive information service and in 1977 work began on its establishment, with the goal of eventual recognition as the worldwide authority for the production, retrieval and dissemination of information on trypanosomiasis and theileriosis. A considerable number of papers were prepared for publication by ILRAD scientific staff in 1977, and many more will appear in 1978. The ILRAD library has been established. It cooperates closely with other such institutions in the collection of relevant information and will be available to all those interested. In 1977, the information service was considerably strengthened by the introduction of literature searches from computerized systems in the United States and Europe. A proposed programme for information dissemination is currently under review, and a variety of means is being discussed. A matter of particular concern to ILRAD is that both other scientific institutions and those working in isolation in the field should be aware of new developments, and that information dissemination should closely support participants in the various training programmes to ensure maximum effectiveness.

FACILITIES



Isolation buildings.



The ILRAD administration wing.

The contract for the main ILRAD facilities was let in April 1976. It is anticipated that the official inauguration and opening of the institute will occur in April 1978. Thus, the main facilities will have been constructed in approximately two years with the final cost being very close to the original estimates.

Included in the main laboratory complex are six modular laboratories including offices and space for trainees. These laboratories are located around a central support core. A central laboratory animal facility is included, as is a necropsy and incineration area. Library, administration, housing and dining facilities are also included. A hostel for trainees, as well as apartments for short-term visitors, make up the ILRAD housing. A training centre, composed of a conference room with translation, projection and other facilities which can be divided into seminar rooms, provides the major teaching and training space. Associated with this is a laboratory for the teaching of general laboratory techniques.

Technician John Kamau staining and mounting tissue in the histopathology laboratory.

Other facilities include a tsetse and a tick unit for investigations involving these two vectors. The animal facilities include pens, sheds, paddocks, a calf-rearing facility, enclosed stable and isolation buildings. All of these are designed as multiuse facilities with flexibility in mind. Additional support activities include a central feed storage barn, vehicle maintenance unit and stores. The latter will be located in the present administrative and laboratory buildings that were on site when the land was acquired from the Kenya Government.

Equipment for the laboratories is modern and oriented to the research indicated in other parts of this report. The facilities and equipment are excellent and represent an institution that is probably unparalleled anywhere else in Africa. ILRAD represents an international resource that should be utilized for research, training and as a focal point for investigations on trypanosomiasis and theileriosis. In addition to being used by ILRAD staff, the facilities are at present and will in the future be utilized by visiting researchers from other institutions in Africa and elsewhere.

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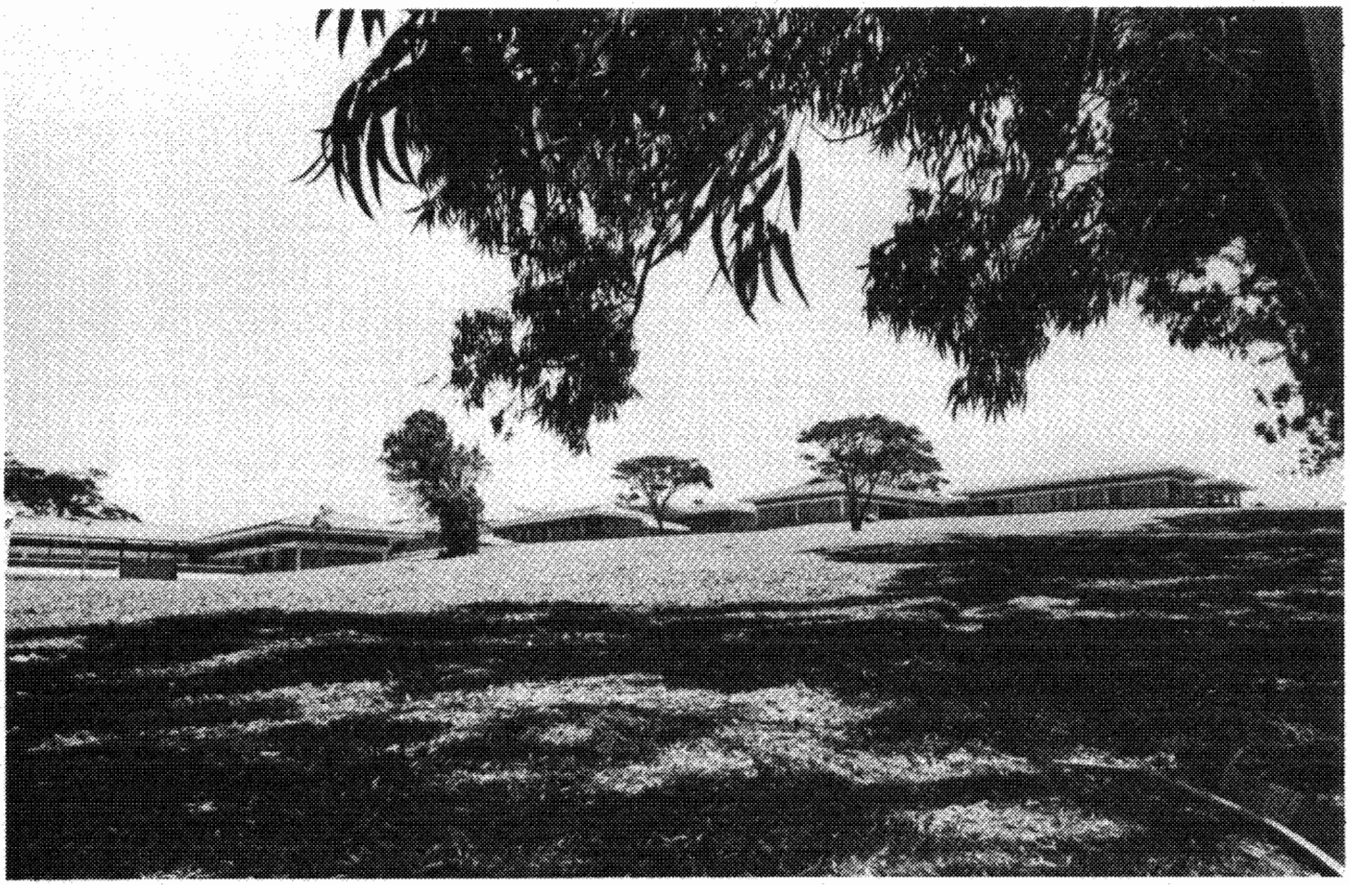
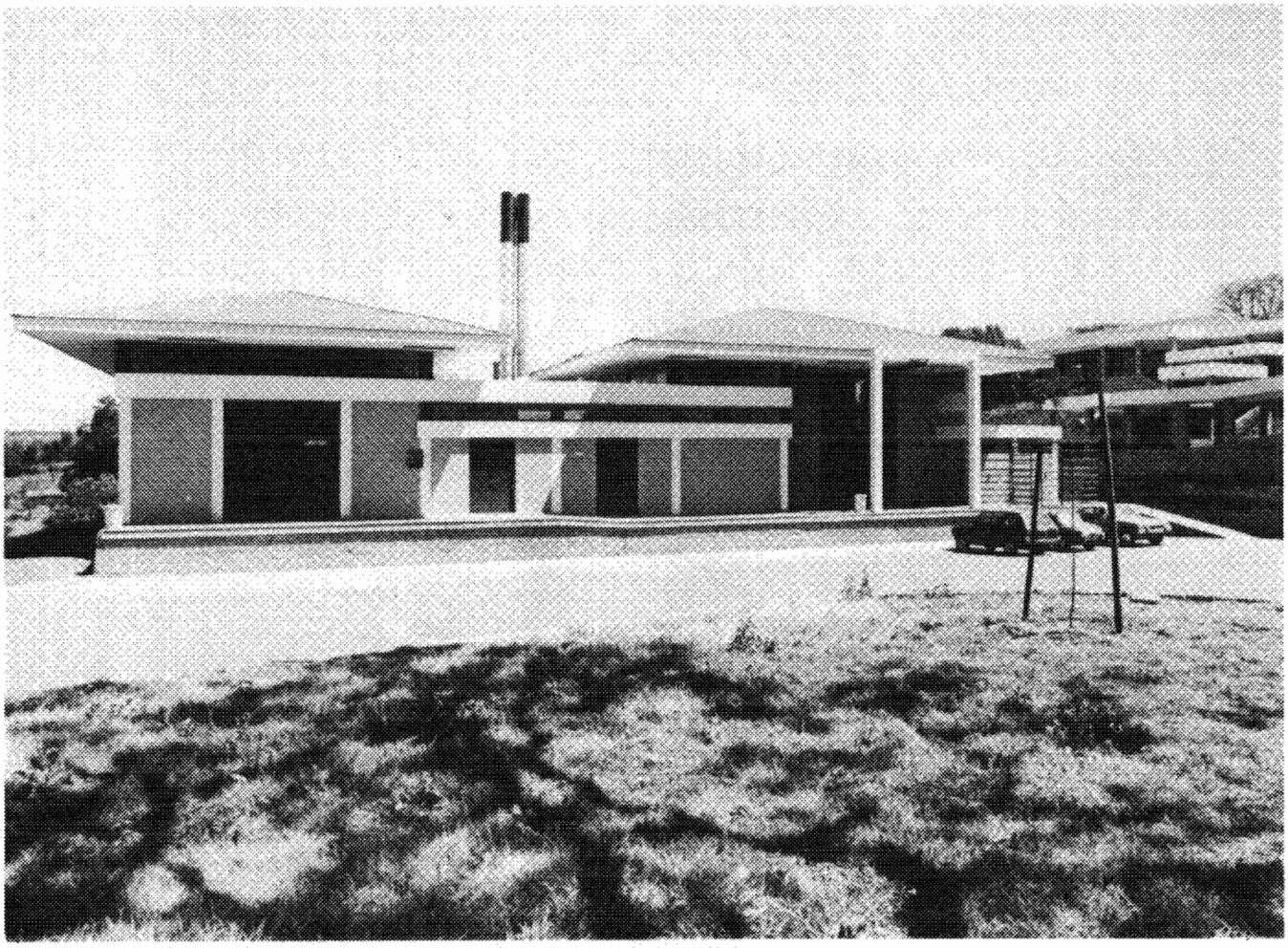


ILRAD grounds.

Necropsy unit.



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Canada
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International Development Association
Kenya
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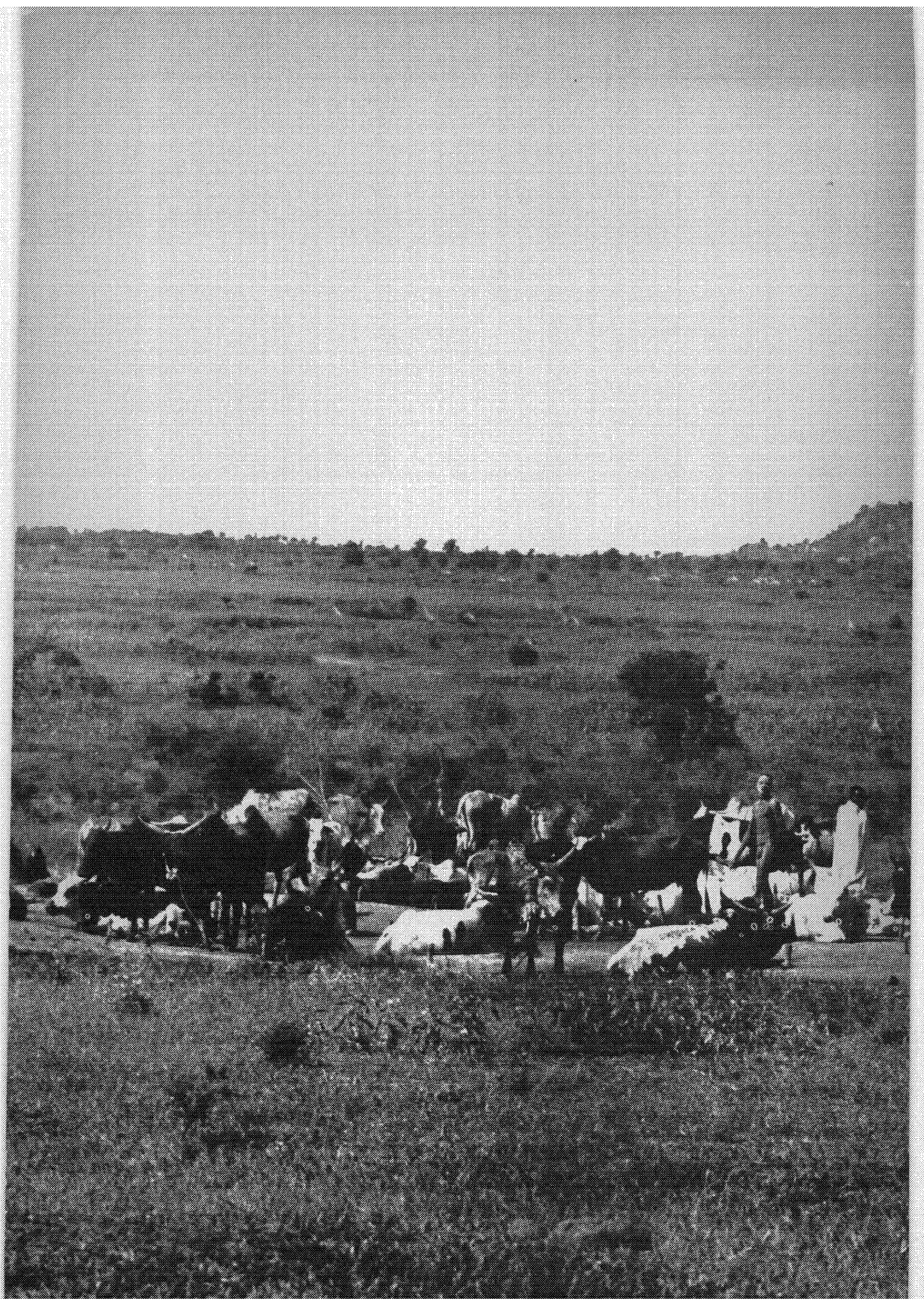
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